



**Substitutos Ósseos Para Regeneração do
Tecido Ósseo: Estudos *In Vivo* e Futuras
Aplicações Clínicas Em Medicina
Veterinária**

Luís Miguel Paiva Benites Da Silva Atayde

Tese De Doutoramento Em Ciências Veterinárias

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**Substitutos Ósseos Para Regeneração do Tecido Ósseo: Estudos
In Vivo e Futuras Aplicações Clínicas Em Medicina Veterinária**

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RESUMO:

Nas últimas décadas verificou-se um alargamento da necessidade de utilização de enxertos ósseos, sendo o tecido ósseo, na atualidade, o segundo tecido mais transplantado, só ultrapassado pelo sangue. Apesar dos autoenxertos serem considerados o enxerto ósseo ideal, em termos de osteocondutividade, osteogenicidade e osteoindutividade com uma histocompatibilidade plena e reações imunológicas adversas mínimas, este tipo de enxerto apresenta alguns pontos críticos, como a necessidade de uma segunda intervenção cirúrgica para recolha do enxerto e a limitação da quantidade de tecido disponível. Outra alternativa é o uso de aloenxertos e xenoenxertos, mas nestes casos existem riscos de transmissão de doenças infectocontagiosas e de reações imunológicas. Estas limitações têm conduzido a uma procura sistemática e muito intensa de biomateriais para serem utilizados como substitutos ósseos sintéticos, fazendo com que a utilização clínica deste tipo de materiais cresça anualmente.

Previamente à utilização dos biomateriais em situações clínicas, deverão ser realizados testes em modelos animais, para avaliar a segurança da sua utilização e avaliar a sua *performance in vivo*. Na primeira fase do trabalho desenvolvido no âmbito desta tese, descreve-se e valida-se um modelo animal, que utiliza o fémur de ovino, para estudar biomateriais na regeneração óssea. Este modelo animal proposto permite realizar amostras em série no mesmo animal, em condições rigorosamente iguais, sendo possível comparar diferentes composições de materiais, com resultados fiáveis. Apesar de neste modelo serem realizados defeitos não críticos em ambos os fémures do animal, foi provado que se consegue avaliar o comportamento dos biomateriais, sendo possível inclusive detetar diferenças entre diferentes biomateriais em estudo. Os defeitos não críticos também possibilitam a observação e a avaliação de todas as fases da cicatrização/regeneração óssea em períodos de implantação curtos. Outra vantagem é a possibilidade da análise em série num mesmo animal, controlando-se assim a variabilidade individual e diminuindo assim o número de animais utilizado na experimentação, sem perder a validade estatística. Para compreender a regeneração óssea no modelo proposto, estudou-se e descreveu-se o processo de cicatrização, sem influência do biomaterial, nos defeitos ósseos criados no fémur

ovino com um diâmetro de 5 mm (chamados defeitos controlo). Uma das problemáticas no estudo dos biomateriais é metodologia usada para a avaliação do comportamento dos biomateriais em estudos *in vivo*. Nestes trabalhos, desenvolveu-se uma metodologia computadorizada semi-automática para realização da análise histomorfométrica (método de segmentação de imagem), que mostrou ser um método reproduzível, fiável, objetivo e mais rápido que os métodos tradicionalmente usados e descritos na bibliografia disponível.

O Bonelike® é um substituto ósseo sintético, desenvolvido na década de 90 por Santos e seus colaboradores (Jha, Santos et al. 1996, Santos, Jha et al. 1996, Knowles, Hastings et al. 2000), composto por dois tipos de materiais, a hidroxiapatite e uma base vítrea, que após serem submetidos a um tratamento térmico, dão origem a uma hidroxiapatite modificada com uma matriz secundária β e α de fosfato de tricálcio. Esta conjugação da hidroxiapatite com o fosfato de tricálcio apresenta uma série de vantagens em relação a cada um dos seus componentes isolados. Apesar destas características da composição química do Bonelike® tornou-se necessário melhorar a sua injectabilidade, para aplicação clínica, principalmente em cirurgias minimamente invasivas. Pretendeu-se alterar a sua arquitectura e a microestrutura dos grânulos, no sentido de melhorar, como referido, a manipulação cirúrgica e aumentar a osteointegração e a regeneração óssea. Assim um dos objetivos deste trabalho foi desenvolver uma nova formulação do Bonelike®, com grânulos esféricos, tendo-se testado para isso duas granulometrias diferentes, 250-500 μm e 500-1000 μm . Estudou-se deste modo, a influência da forma física, estrutura e microestrutura das diferentes formulações e granulometrias de Bonelike® na regeneração óssea. Os resultados obtidos comprovaram a influência da forma, tamanho, estrutura e microestrutura do substituto ósseo no comportamento de um enxerto ósseo. Verificou-se que o Bonelike® esférico tem uma microestrutura com porosidades maiores quando comparada com a forma poligonal, que favorecem a osteocondutividade, osteointegração e a absorção do biomaterial durante o processo de regeneração, adaptando-se melhor ao crescimento ósseo. Além das vantagens apresentadas na *performance* do biomaterial, foi constatado que o Bonelike® esférico apresenta uma maior facilidade de aplicação através de seringa, com uma injectabilidade melhorada em relação ao formato poligonal.

Por último pretendeu-se aplicar o Bonelike® esférico em casos clínicos de Medicina Veterinária. Este biomaterial foi aplicado em 3 casos clínicos, de 2 cavalos e de um burro, para promover a artrodese cirúrgica das articulações com patologia. Os casos clínicos descritos reportam-se a um cavalo adulto com uma subluxação da articulação interfalângica proximal, a um burro com 10 dias de idade com uma deformidade flexora severa da articulação metacarpofalângica e a um cavalo adulto com artrose e instabilidade das articulações intertársica distal e tarsometatársica. Com a exceção do caso clínico da deformidade flexora, onde surgiram complicações pós-cirúrgicas não relacionadas com a aplicação intra-articular de Bonelike®, verificou-se que a fusão óssea das articulações foi promovida corretamente. Em nenhum dos casos clínicos foram detetadas reações adversas locais ou sistémicas à utilização do Bonelike®. Concluiu-se que Bonelike® esférico é um substituto ósseo que pode ser usado em casos clínicos de artrodese para preencher espaços vazios, aumentar a estabilidade articular e para promover e acelerar a osteorregeneração.

ABSTRACT:

In the last decades, there has been an increasing need for the use of bone grafts, being currently the second most transplanted tissue, surpassed only by blood. Although autograft is considered to be the ideal bone graft, particularly in terms of osteoconductivity, osteogenecity and osteoinductivity, with full histocompatibility and minimal adverse immunological reactions, but this type of graft also shows some critical points, like the need of a second surgical intervention to collect the graft and limitations to the quantity of available bone tissue. An alternative is the use of allografts and xenografts, but these involve the risks of transmission of infectious diseases and immunological reactions. These limitations have led to a systematic and very strong demand for biomaterials, to be used as synthetic bone substitutes, and the clinical use of these materials is increasing every year. Prior to the use of biomaterials in clinical situations, these must be tested in animal models in pre-clinical studies, to evaluate the safety of their use and their *in-vivo performance*.

During the first phase of the work of this thesis, it is described and validated an animal model, which uses the femur of a sheep to study biomaterials on bone regeneration. The proposed animal model enables serial sampling in the same animal, under the exact same conditions, allowing comparison of different material compositions, with reliable results. Although defects performed on both femurs of the animal were non-critical, it was proved that it is possible to evaluate the behaviour of biomaterials and even detect differences between the different studied materials. Non-critical defects also allow observing and evaluating all healing/regeneration phases over short implantation periods. Another advantage is the possibility of doing serial analysis, in the same animal, thus controlling individual variability and reducing the number of animals used on experiments, without losing statistical validity. To understand bone regeneration on the proposed model, the healing process was studied and described without the influence of biomaterial, on 5mm diameter bone defects created on the sheep's femur (the so called control defects). One of the issues, when studying biomaterials, is the method used to evaluate the behaviour of biomaterials on *in vivo* studies. For these type of studies, a computerized and semi-automatic methodology has been developed to perform histomorphometric analysis (image segmentation method), which has proven to be

reproducible, feasible, objective and faster than the methods traditionally used and described in available literature.

Bonelike® is a synthetic bone substitute developed in the 90's by Santos and collaborators (Jha, Santos et al. 1996, Santos, Jha et al. 1996, Knowles, Hastings et al. 2000), composed by two raw of materials, hydroxyapatite and a vitreous base, which are subjected to heat treatment, giving rise to a modified hydroxyapatite, with a β and α secondary matrix of tricalcium phosphate. This combination of hydroxyapatite and tricalcium phosphate, presents several advantages in relation to each of its isolated components. Despite the characteristics of the chemical composition of Bonelike®, it became necessary to improve its injectability for clinical application, especially in minimally invasive surgeries. The intention was to change its architecture and granule microstructure, to improve surgical manipulation and enhance osteointegration and bone regeneration. Therefore, one of the objectives of this work was to develop a new Bonelike® formulation, with spherical granules, and for that purpose, two different particle sizes were tested, 250-500 μm and 500-1000 μm . With this, it was studied the influence of physical shape, structure and microstructure of different formulas and granule size of Bonelike® on bone regeneration. The results obtained have proven the influence of shape, size, structure and microstructure of the bone substitute on the behaviour of a bone graft. It was found that spherical Bonelike® shows a microstructure with larger porosity, when compared to polygonal shaped Bonelike®, which favours osteoconductivity, osteointegration and material absorption during the regeneration process and adapts better to bone growth. Besides the advantages presented on the biomaterial *performance*, it was found that the spherical Bonelike® is easier to apply with a syringe and has improved injectability, when compared to the polygonal format. Finally, it was sought the application of spherical Bonelike® in clinical cases of Veterinary Medicine. This biomaterial was applied on 3 clinical cases, of 2 horses and a donkey, to promote surgical arthrodesis on joints with pathology with clinical indication for arthrodesis. The described clinical cases report to an adult horse with sub-luxation proximal interphalangeal joint, a 10-day-old donkey with severe flexor deformity of the metacarpophalangeal joint and another adult horse with arthrosis and a slight biomechanical instability of the distal intertarsal and the tarsometatarsal joint. Except for the clinical case of the donkey with flexor deformity, that suffered post-surgical complications, unrelated to the intra-articular application of Bonelike®,

it was found that bone fusion was correctly promoted. None of the clinical cases showed local or systemic adverse reactions with the use of Bonelike®. It is concluded that spherical Bonelike® is a bone substitute that can be used in clinical cases of arthrodesis, to fill empty spaces, increase joint stability and to promote and accelerate osteo-regeneration.

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Capítulo 1

Introdução

1 – INTRODUÇÃO:

O osso é um tecido que apresenta propriedades muito importantes no organismo, tais como o suporte do corpo, proteção dos órgãos internos e o armazenamento e troca de iões. O tecido ósseo tem uma boa capacidade regenerativa, mas apesar disso esta capacidade regenerativa pode não ser suficiente, em certas lesões extensas, sendo necessário encontrar alternativas para promover a cicatrização óssea (Kamitakahara, Ohtsuki et al. 2008). Com o aumento da esperança média de vida, e o envelhecimento da população, em conjunto com a crescente atividade desportiva com risco de trauma, levou ao incremento do aparecimento de doenças ortopédicas (Bohner 2010). Assim, nas últimas décadas verificou-se um alargamento da necessidade de utilização de enxertos ósseos em medicina humana, essa necessidade verificou-se também na medicina veterinária, sendo o tecido ósseo, na atualidade, o segundo tecido mais transplantado, só ultrapassado pelo sangue (Mahan and Carey 1999, Perry 1999, Lesser 2002, Giannoudis, Dinopoulos et al. 2005, Bohner 2010, Dorozhkin 2010).

O recurso à utilização de enxertos ósseos está indicado em várias situações, como em complicações pós traumáticas do aparelho músculo-esquelético, com atraso de união entre os bordos ósseos, falhas ósseas; preenchimento após remoção de tumores ósseos; fusões da coluna vertebral (Giannoudis, Dinopoulos et al. 2005). O enxerto ósseo, ao preencher o espaço vazio, além de aumentar a resistência local, forma uma matriz extracelular para suportar e conduzir o crescimento ósseo. A imobilização e recuperação do formato anatómico normal são outras funções importantes do enxerto ósseo. (Kolk, Handschel et al. 2012). Atualmente desenvolvem-se técnicas para os enxertos ósseos transportarem e libertarem de forma controlada, substâncias ativas, como medicamentos, promotores de crescimento ósseo e antibióticos e ainda permitirem associar terapias génica e celular (Fischer, Kolk et al. 2011, Kolk, Haczek et al. 2011, Kolk, Handschel et al. 2012, Ben-David, Srouji et al. 2013, Bormann, Schwabe et al. 2014).

Osteogénese, osteoindução, osteocondução e osteointegração são as propriedades que um enxerto ósseo deve apresentar para ser utilizado em clínica

(Giannoudis, Dinopoulos et al. 2005, Shi 2005). Osteogénese implica a existência de células osteoprogenitoras vivas no enxerto, que podem proliferar e diferenciar-se em osteoblastos e osteócitos. A osteoidução é a capacidade do enxerto estimular as células mesenquimatosas indiferenciadas, dos tecidos vizinhos ao implante, de se diferenciarem em osteoblastos e começarem a produzir tecido ósseo. Osteocondução é a capacidade do enxerto encaminhar a formação de vasos sanguíneos com a consequente criação de novo osso através do enxerto. Por último, osteointegração refere a capacidade do enxerto se interligar ao osso original (Erbe, Marx et al. 2001, LeGeros, Lin et al. 2003, Giannoudis, Dinopoulos et al. 2005, Shi 2005, Salgado, Sathler et al. 2011).

A utilização do autoenxerto, enxerto recolhido do próprio paciente, é considerada clinicamente como sendo a técnica de eleição, dadas as suas propriedades inerentes de osteocondutividade, osteogenicidade e osteoindutividade com uma histocompatibilidade plena e reações imunológicas adversas mínimas (Perry 1999, Bohnert 2010, Nandi, Roy et al. 2010, Kolk, Handschel et al. 2012). Apesar destas características únicas, os autoenxertos têm sido substituídos, de forma crescente, por outros tipos de substitutos ósseos, devido a alguns pontos críticos da sua utilização, nomeadamente, a elevada taxa de morbilidade, de cerca de 20%, associada à 2ª cirurgia para a obtenção do autoenxerto, com o consequente prolongamento do tempo de anestesia; a limitada quantidade disponível de tecido osso e as possíveis complicações associadas à recolha do autoenxerto (Perry 1999, Giannoudis, Dinopoulos et al. 2005, Bohnert 2010, Kolk, Handschel et al. 2012). Outra alternativa possível é o uso de aloenxertos, enxerto recolhido de um indivíduo da mesma espécie. Mas, neste caso a transmissão de doenças infecto-contagiosas e as reações imunológicas adversas, quer em Medicina Humana quer em Medicina Veterinária, constitui um perigo real (Tomford 1995, Boyce, Edwards et al. 1999, Bohnert 2010, Salgado, Sathler et al. 2011). O controlo meticuloso dos dadores, das técnicas de processamento e dos métodos de esterilização diminui os riscos mas não os elimina completamente (Scarborough, While et al. 1995, Nandi, Roy et al. 2010, Salgado, Sathler et al. 2011). Além disso, os métodos de processamento e esterilização dos aloenxertos diminuem tanto as capacidades mecânicas, como osteoindutivas deste tipo de enxerto (Giannoudis, Dinopoulos et al. 2005, Kolk, Handschel et al. 2012). Outra possibilidade será a utilização de

xenoenxertos, enxertos recolhidos de indivíduos de espécie diferente, persistindo a desvantagem da possibilidade de transmissão de doenças infecto contagiosas e incrementando-se os riscos de reações imunológicas adversas (Bohner 2010, Vertenten, Gasthuys et al. 2010, Salgado, Sathler et al. 2011, Kolk, Handschel et al. 2012). As dificuldades de manipulação e de adaptação ao formato da lesão poderá ser outra limitação do uso dos autoenxertos, aloenxertos e xenoenxertos (Bohner 2010).

Enxertos ósseos sintéticos:

Nas últimas décadas, estas limitações têm conduzido a uma procura sistemática e muito intensa de biomateriais para serem utilizados como substitutos ósseos sintéticos, fazendo com que a utilização clínica deste tipo de materiais cresça anualmente (Lane and Khan 2000, Giannoudis, Dinopoulos et al. 2005). Biomaterial pode ser definido como qualquer substância usada para tratar, substituir ou aumentar qualquer tecido vivo, interagindo com esse tecido sem causar reações adversas no organismo e nos seus componentes (Helmus, Gibbons et al. 2008, Parida, Behera et al. 2012, Dorozhkin 2013). Existem inúmeros tipos de enxertos ósseos sintéticos que poderão ser divididos em metais, polímeros, cerâmicos e compósitos (Bohner 2010, Dorozhkin 2010, Bohner, Galea et al. 2012).

Um enxerto ósseo ideal deveria ser reabsorvido, conferir uma resistência semelhante ao osso cortical, ter propriedades osteocondutivas e de osteoindução, ser fácil de manipular, esterilizável e não causar reações adversas; contudo não existe nenhum biomaterial que consiga englobar todas estas propriedades (McAuliffe 2003, Giannoudis, Dinopoulos et al. 2005, Bohner, Galea et al. 2012, Chen, Zhu et al. 2012, Parida, Behera et al. 2012).

Metais:

Os Metais, como o titânio, cobalto, cromo e aço inox, são usados preferencialmente na fixação de fraturas, porque conferem estabilidade mecânica, contudo este tipo de materiais não apresentam propriedades de reabsorção, osteocondução e osteoindução. Outra desvantagem é a ausência

de elasticidade, com uma consequente concentração de forças no local do implante e, além disso, poderão estar sujeitos a corrosão, com libertação de substâncias tóxicas (Perry 1999, Shi 2005, Rodríguez-González 2009, Srivastav 2011).

Polímeros:

Os polímeros são usados em inúmeras situações tais como elementos de fixação óssea, material de sutura, próteses, material dentário, estruturas porosas tridimensionais para preenchimento ósseo, membranas e sistemas de libertação de fármacos (Griffith 2000, Nair and Laurencin 2007, Kolk, Handschel et al. 2012, Lasprilla, Martinez et al. 2012, Parida, Behera et al. 2012, Tian, Tang et al. 2012).

Cerâmicos:

Os cerâmicos são sólidos não metálicos inorgânicos preparados pela ação do calor e subsequente arrefecimento (Hamadouche and Sedel 2000, Dorozhkin 2013). Fazem parte deste grupo de materiais, os fosfatos de cálcio (hidroxiapatite e fosfato de tricálcio), o sulfato de cálcio, derivados do coral, alumina (óxido de alumínio) e zircónica.

A alumina e a zircónica têm vindo a ser usados desde os anos 60, em várias aplicações clínicas como implantes dentários e ortopedia. São duros, com baixos coeficientes de fricção e resistentes à corrosão, mas são relativamente inertes sem formarem ligações diretas ao osso novo (Hamadouche and Sedel 2000, Hallman, Cederlund et al. 2001).

O carbonato de cálcio do esqueleto do coral pode ser convertido em hidroxiapatite por processos de sinterização a altas temperaturas (Gazdag, Lane et al. 1995, Tay, Patel et al. 1999) tendo, o primeiro método para a formação dos cerâmicos derivados do coral, sido descrito em 1974 (Roy and Linnehan 1974).

O sulfato de cálcio foi o primeiro substituto ósseo sintético a ser usado, ainda no séc. XIX (Tay, Patel et al. 1999). Os cerâmicos de sulfato de cálcio são reabsorvidos rapidamente, não permitindo que ocorra a cura do defeito antes do seu desaparecimento (McAuliffe 2003).

Os fosfatos de cálcio como a hidroxiapatite (HA) e os fosfatos de tricálcio, são substitutos ósseos usados amplamente, pois apresentam uma estrutura semelhante à parte mineral do osso, mas apesar de serem duros são frágeis

com fraca estabilidade mecânica. A hidroxiapatite é bioactiva, formando ligações diretas ao osso novo e os fosfatos de tricálcio são reabsorvidos.

Compósitos:

O termo compósito normalmente é usado para o tipo de material onde existem fases distintas numa escala maior que a atómica (Parida, Behera et al. 2012), em que a matriz de um determinado material é preenchida por outro tipo de material (Rodríguez-González 2009). As propriedades deste produto final vão ser diferentes dos compostos originais, obtendo-se assim um material melhorado com a conjugação das propriedades dos seus componentes (Rodríguez-González 2009, Parida, Behera et al. 2012).

Interação dos biomateriais com o organismo:

Desde os primórdios da utilização de biomateriais surgiram problemas, com a maneira como estes vão interagir com os tecidos envolventes. Dessas interações, poderão surgir vários tipos de complicações, como a rejeição do material pelo organismo, com morte do tecido envolvente ao local do implante; corrosão do material com libertação de substâncias tóxicas; fragilização do material, ao longo do tempo, com consequente perda de função (Hennessy and Ben-Nissan 2004, Srivastava 2011). O requisito principal de um biomaterial é ser biocompatível, propriedade em que o material estranho apresenta uma resposta adequada quando implantado no tecido vivo, sem causar danos nos tecidos envolventes (Srivastava 2011). Quando um biomaterial é implantado no organismo vivo, desencadeiam-se uma série de reações que estão dependentes do tipo de material utilizado. Os biomateriais poderão ser classificados, conforme a resposta que desencadeiam no organismo, como tóxicos, bioinerte, bioreabsorvível e bioactivo (**Figura 1 e Figura 2**) (Hennessy and Ben-Nissan 2004, Srivastava 2011). O material tóxico provoca uma reacção adversa nos tecidos vizinhos ao implante, com morte celular (Srivastava 2011). Bioinerte corresponde ao tipo de biomaterial que desencadeia uma reacção mínima com o tecido envolvente, como por exemplo o aço inoxidável, titânio, o óxido de alumínio e a zircónia; nestas situações uma cápsula fibrosa fina, não aderente, forma-se á

volta do implante, não havendo uma ligação direta (mineralizada) entre o osso e o biomaterial. Bioreabsorvível é o tipo de material que se dissolve (absorve) ao longo do tempo e é substituído por osso, como por exemplo os fosfatos tricálcico. Bioativo o tipo de material que interage com o tecido envolvente, existindo reações de troca de iões com os fluidos envolventes e a formação de apatites carbonadas, formando ligações fortes e diretas entre o osso e o biomaterial com uma interface única e forte, como por exemplo a hidroxiapatite (LeGeros, Lin et al. 2003, Heness and Ben-Nissan 2004, Kamitakahara, Ohtsuki et al. 2008).

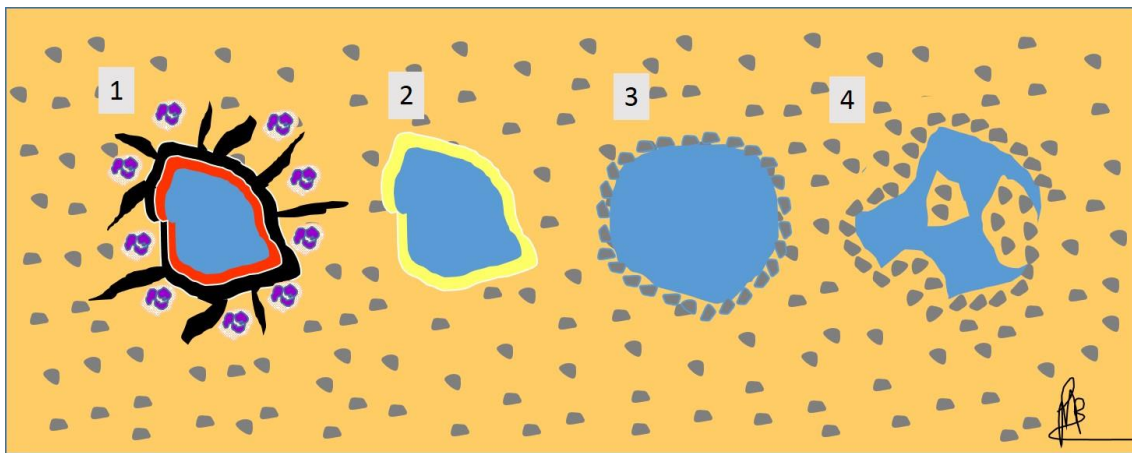


Figura 1 - Representação esquemática da resposta de um biomaterial no organismo vivo. 1 – Toxico – reação inflamatória exuberante com morte celular; 2 – Bioinerte – reação mínima com a possibilidade de formação de uma cápsula fibrótica; 3 – Bioativo – integra-se com o tecido envolvente; 4 – Bioreabsorvível – absorve / dissolve ao longo do tempo.

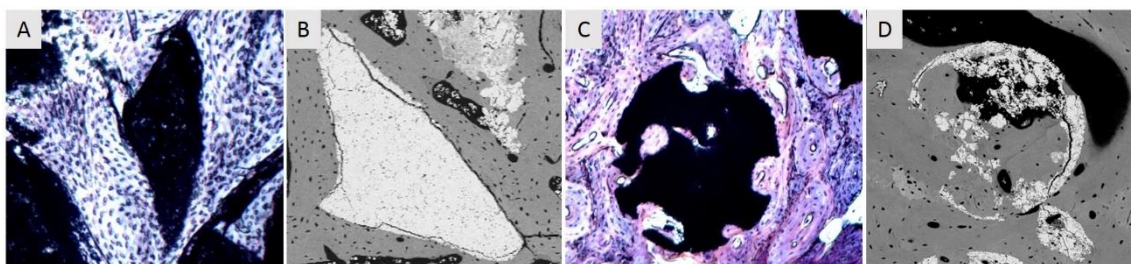


Figura 2 – Imagens histológicas coradas com Solochrome (A e C) e de microscopia eletrônica de varrimento (SEM) (B e D). Material Bioinerte (A e B) e material Bioreabsorvível (C e D)

Fosfatos de cálcio:

Os fosfatos de cálcio é um tipo de material sintético que tem sido amplamente estudado e desenvolvido nas últimas décadas (Bohner 2010, Dorozhkin 2013). Este tipo de materiais mimetiza a fase mineral do osso, devido à similaridade destes compostos com a apatite natural do tecido ósseo (McAuliffe 2003, Salgado, Sathler et al. 2011). A principal vantagem dos fosfatos de cálcio é a não interferência, por parte dos íons de cálcio e fosfato, com a função celular e a fisiologia dos tecidos envolventes. Além disso, durante a dissolução deste material, a liberação deste tipo de íons poderá funcionar como catalisador para a formação de osso e a precipitação de camadas de apatite adjacente à superfície do material (Salgado, Sathler et al. 2011).

Hidroxiapatite sintética (HA):

A HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, é um dos fosfatos de cálcio utilizado com mais frequência como substituto ósseo. Apresenta uma estrutura similar à fase mineral do osso, com propriedades de osteocondução, biocompatibilidade e bioatividade, ligando-se diretamente ao osso sem mediação de tecido fibrótico (Stevenson 1999, Burg, Porter et al. 2000, Keating and McQueen 2001, Vaccaro 2002, Damien and Revell 2004). A HA é um composto bioinerte com uma boa osteointegração, mas frágil e com taxas de reabsorção baixas (5 a 15% por ano) (Fleming, Cornell et al. 2000, Giannoudis, Dinopoulos et al. 2005). O facto de a HA não apresentar propriedades mecânicas que confirmem resistência, em conjunto com a longa permanência no organismo, sem que haja uma remodelação e substituição, comprometem a restauração da resistência normal do osso, fazendo com que o osso permaneça por longos períodos fragilizado e sujeito a fraturas (Boyan, McMillan et al. 2003, Giannoudis, Dinopoulos et al. 2005). Estas limitações levaram ao desenvolvimento de fórmulas para melhorar a reabsorção da HA. Estas alterações consistem em modificar a HA, combinando-a com substâncias que incrementem a sua taxa de absorção (Passuti, Daculsi et al. 1989, Bucholz 2000, Keating and McQueen 2001).

Fosfato de tricálcio (FTC):

Outros fosfatos de cálcio muito utilizados são os fosfato de tricálcio ($\text{Ca}_3[\text{PO}_4]_2$) (FTC), que contêm 39 % de cálcio e 20 % de fósforo, composição esta semelhante à parte mineral do osso (Erbe, Marx et al. 2001, Hak 2007). Os FTC são mais solúveis que a HA, com uma taxa de absorção *in vivo* maior (Daculsi, LeGeros et al. 1989, Koerten and Van der Meulen 1999, Kamitakahara, Ohtsuki et al. 2008). A taxa de reabsorção de um biomaterial deverá ser ao ritmo da formação de osso novo. Taxas de absorção lentas fazem com que o material permaneça no local e impeça o crescimento de osso novo, como o exemplo da HA. Pelo contrário no caso dos FTC a taxa de absorção é mais rápida que o ritmo do crescimento de osso novo, levando que a massa de osso novo não preencha o espaço deixado pela reabsorção do FTC, fragilizando o local do implante. (Jarcho 1981, Hollinger, Brekke et al. 1996, Moore, Graves et al. 2001). As interligações entre o implante e o osso novo são fundamentais para fortalecer e estabilizar o local da lesão. Idealmente o osso novo deve ligar-se ao implante por ligações diretas com formação de apatites carbonadas numa interface única. As elevadas taxas de absorção dos FTC poderão comprometer a formação destas ligações diretas (Kotani, Fujita et al. 1991, Kamitakahara, Ohtsuki et al. 2008).

Existem 2 fases cristalinas de fosfato de tricálcio, alfa (α -FTC) e a beta (β -FTC), a fase β é mais estável e apresenta um ritmo de absorção mais lento (Chow 1991, Kamitakahara, Ohtsuki et al. 2008, Uchino, Yamaguchi et al. 2010). A elevada taxa de solubilidade da α -FTC poderá em enxertos ósseos permitir a libertação medicamentos ou substâncias ativas como fatores de crescimento que promovam a osteogénese (Uchino, Yamaguchi et al. 2010).

Fosfatos de cálcio bifásicos:

Um biomaterial ideal deverá ligar-se diretamente ao osso, e ser reabsorvido a uma taxa igual à de osso novo produzido, para assim haver uma integração do implante e uma substituição gradual deste por osso novo, sem que no período que medeia a substituição do osso novo pelo implante a estrutura fique fragilizada. Assim, para colmatar as desvantagens da deficiente absorção

da HA e da rápida absorção dos FTC, desenvolveu-se o conceito de material bifásico, que resulta da combinação destas duas substâncias (Nery, LeGeros et al. 1992, Brown, McAfee et al. 2010, Yang, Unursaikhan et al. 2013). Este tipo material bifásico permite adaptar o ritmo de absorção ao crescimento ósseo, alterando a proporção de HA/FC. Várias proporções da mistura HA/FC têm sido propostas, com o ritmo de reabsorção inversamente proporcional a este rácio (Daculsi, LeGeros et al. 1989, Yamada, Heymann et al. 1997, LeGeros, Lin et al. 2003). Outra vantagem deste tipo de compostos bifásicos, advém da possibilidade da absorção mais rápida do FTC levar à precipitação de iões de fosfato de cálcio, que favorecem a formação de ligações diretas entre o composto bifásico e o osso novo (Daculsi, LeGeros et al. 1989, Kamitakahara, Ohtsuki et al. 2008). Para melhorar a bioatividade e a regeneração óssea, estes compostos bifásicos podem ser suplementados com oligoelementos como o magnésio (Yasukawa, Ouchi et al. 1996), o flúor (Jha, Best et al. 1997), o sódio (De Maeyer and Verbeeck 1993), o Zinco e o Silício (Kamitakahara, Ohtsuki et al. 2008), obtendo-se assim uma composição química mais semelhante à fase mineral do osso.

Bonelike®:

Santos e colaboradores (Jha, Santos et al. 1996, Santos, Jha et al. 1996, Knowles, Hastings et al. 2000) desenvolveram um método de produção, de um substituto ósseo sintético, composto por dois tipos de materiais, a HA e uma base vítrea, que após serem submetidos a um tratamento térmico, dão origem a uma HA modificada com uma matriz secundária β e α -FTC. Este produto final já foi patenteado e registado como Bonelike® (Jha, Santos et al. 1996, Santos, Jha et al. 1996, Knowles, Hastings et al. 2000), com uma composição final determinada por difração de raio x e análise por método de Rietveld (Lobato, Hussain et al. 2005). Este material poderá ser considerado como um fosfato de cálcio bifásico ou trifásico caso β -FTC e α -FTC sejam consideradas separadamente. As fases β e α de FTC são dispersadas homogeneamente na matriz de HA devido à presença de uma fase líquida durante o processo de sinterização, resultando uma microestrutura com melhoramento das

propriedades mecânicas e uma bioactividade superior, relativamente à HA pura. Apesar de se iniciar o processo com duas matérias-primas diferentes, após o tratamento térmico, a fase vítrea desaparece, sendo o produto final, Bonelike®, uma matriz de HA modificada com fases secundárias β e α -FTC (Santos, Knowles et al. 1994, Lopes, Knowles et al. 1998, Lopes, Knowles et al. 2000). Outra vantagem desta metodologia é que as proporções de HA/FTC podem ser adaptadas, alterando a temperatura de sinterização, a quantidade e a composição da base vítrea e a presença de iões durante a preparação como o flúor, o magnésio ou o zinco. Podendo-se assim controlar a resistência mecânica e a taxa de reabsorção do produto final, sabendo-se que o FTC é mecanicamente mais frágil que HA e apresenta uma taxa de reabsorção mais elevada (LeGeros, Parsons et al. 1988, Daculsi, LeGeros et al. 1989, Piattelli, Scarano et al. 1996, LeGeros, Lin et al. 2003, Hak 2007). Além disto, a inclusão da base vítrea durante o processo de produção permite a incorporação de diferentes iões na matriz de HA, como o flúor, magnésio e o sódio, aproximando-se assim a composição final do Bonelike®, da composição da fase mineral do osso (Lopes, Santos et al. 2001).

O Bonelike® foi testado em vários testes *in vitro* (Silva, Santos et al. 1998, Ferraz, Fernandes et al. 1999, Ferraz, Fernandes et al. 2001, Silva Marques, Gomes et al. 2009). Ferraz e colaboradores testaram a citocompatibilidade do Bonelike® com culturas celulares obtidas por punção aspirativa de medula óssea humana, e num desses trabalhos foi verificado que os osteoblastos proliferaram e secretaram uma matriz extracelular, que mineralizou posteriormente (Ferraz, Fernandes et al. 1999). Noutro trabalho, também utilizando culturas celulares obtidas por punção aspirativa de medula óssea humana, foi verificado que a composição do Bonelike® tinha melhores *performances* do que a HA isolada, na proliferação osteoblástica e na formação de matriz extracelular mineralizada (Ferraz, Fernandes et al. 2001). Silva Marques e colaboradores, concluíram, através de testes de viabilidade celular, com culturas de células endoteliais humanas, que o Bonelike® permitia a adesão e expansão destas células, e também a subsequente proliferação com a expressão fenotípica de células endoteliais, sugerindo um potencial relevante para a utilização na engenharia de tecidos (Silva Marques, Gomes et al. 2009). Também foram realizados vários testes *in vivo* em modelos animais como o coelho e a ovelha (Lopes, Santos et

al. 2001, Lobato, Hussain et al. 2006, Cortez, Silva et al. 2011). Lopes e colaboradores realizaram o primeiro teste *in vivo* na tíbia de coelhos, onde foi comparado o comportamento do Bonelike® com a HA, onde verificou que o osso se formava mais rapidamente à volta dos implantes de Bonelike®, concluindo que este era mais osteocondutivo relativamente à HA (Lopes, Santos et al. 2001). Lobato e colaboradores também realizaram testes em coelhos, criando defeitos circulares no fémur e preenchendo-os com grânulos poligonais de Bonelike® com 150 a 250 µm, verificando que ao fim de 12 semanas a superfície do Bonelike® estava quase completamente rodeada por osso novo, concluindo que este tipo de enxerto era altamente osteocondutivo e que era um material com bom potencial para ser usado em casos clínicos de cirurgia reconstrutiva e maxilo-facial (Lobato, Hussain et al. 2006). Cortez e colaboradores, utilizaram o fémur de ovino, para testar a aplicação de Bonelike® em forma de grânulos poligonais de 150 a 250 µm com um veículo de sulfato de cálcio, onde confirmou as propriedades osteocondutivas do Bonelike® e verificou que a combinação do Bonelike® com o sulfato de cálcio permitia a fixação e estabilização dos grânulos Bonelike® quando aplicado em defeitos volumosos (Cortez, Silva et al. 2011). O Bonelike® foi ainda utilizado em ensaios clínicos em humanos. Gutierres e colaboradores implantaram na parte lateral da tíbia, cilindros de Bonelike® quando realizava a osteotomia, no tratamento cirúrgico, da osteoartrite compartimental do joelho. Os cilindros foram removidos 6, 9 e 12 meses após a sua implantação, no momento em que se realizava a segunda cirurgia para tratamento desta patologia. Estas amostras foram sujeitas a análises histológicas e de microscopia eletrónica de varrimento (SEM). Com este estudo os autores concluíram que este tipo de material era adequado para utilização em humanos, apresentando propriedades osteocondutivas e uma osteointegração adequada (Gutierres, Hussain et al. 2005, Gutierres, Hussain et al. 2006, Gutierres, Lopes et al. 2008). O Bonelike® também foi utilizado em várias situações clínicas de medicina humana como, no preenchimento de defeitos ósseos após a extração de quistos, em implantologia, após a remoção cirúrgica de dentes inclusos, no preenchimento de quistos ósseos de duas ou mais paredes, assim como em bifurcações ou trifurcações de dentes, na elevação óssea do seio maxilar e em inúmeras aplicações ortopédicas (Gutierres, Hussain

et al. 2005, Gutierres, Hussain et al. 2006, Lobato, Hussain et al. 2006, Gutierres, Dias et al. 2007, Gutierres, Lopes et al. 2008, Sousa, Lobato et al. 2008).

Importância da morfologia dos biomateriais:

O comportamento biológico dos cerâmicos, além de estar dependente da composição química, também é influenciado pelas suas características físicas e morfológicas (Bohner 2010). No mercado estão disponíveis vários cerâmicos com formas diversas, contrastando tanto nas dimensões como no formato. Um dos pontos críticos do preenchimento de defeitos ósseos com biomaterial é a adaptação ao defeito. Poderá conjecturar-se que blocos com um formato idêntico ao defeito, como por exemplo as cunhas de Bonelike®, usadas para o tratamento do *valgus* na osteoartrite compartimental do carpo (Gutierres, Dias et al. 2007), seriam uma situação ideal. Mas infelizmente na maior parte das situações clínicas, especialmente as traumáticas, os blocos não têm o mesmo formato que o defeito. Assim, e por outro lado, a possibilidade do uso de fórmulas granulares em multipartículas permite a adaptação a qualquer geometria de defeito, especialmente quando associadas a um veículo adequado. Estas formas granulares mantêm um espaço entre as partículas (interporosidade), que é invadido por uma rede vascular, aumentando assim a capacidade de crescimento óssea e de reabsorção do implante (Bohner, Tadier et al. 2013).

O acesso cirúrgico e a manipulação dos tecidos para reconstrução do defeito, poderá ser outro aspeto a ter em atenção no desenvolvimento de substitutos ósseos sintéticos, não só pela morbilidade pós-cirúrgica que daí pode advir, como também das complicações inflamatórias, com alterações circulatórias, associadas às incisões e manipulações extensas. Com isto desenvolveu-se o conceito da aplicação de biomateriais com técnicas cirúrgicas minimamente invasivas, através de pequenas incisões com pouca manipulação de tecidos (Dupraz, Delecrin et al. 1998, Bohner 2010). Para cumprir com este objetivo torna-se imperativo o desenvolvimento de biomateriais, onde seja possível a aplicação através de injeção, com auxílio de uma seringa. Formas granulares com tamanho adequado para passar pelo bico da seringa cumprem este requisito. O formato dos grânulos também pode simplificar esta tarefa, pois

o formato esférico facilita a fluidez do biomaterial na extrusão, estando os grânulos menos propensos a compactarem (Ishikawa, Matsuya et al. 2004, Böhner, Tadier et al. 2013).

A arquitetura do material com a presença de poros, macroporos, microporos e poros interconectados é outra característica crucial dos substitutos ósseos, pois este tipo de estrutura permite a invasão de vasos sanguíneos, osteoblastos, células endoteliais e células com potencial regenerativo locais, com células estaminais mesenquimatosas presentes em alguns tecidos, bem como o aporte de nutrientes e a remoção de subprodutos do metabolismo celular, promovendo assim a invasão óssea e a reabsorção do biomaterial (Hing 2005, Karageorgiou and Kaplan 2005, Böhner 2010, Sanzana, Navarro et al. 2013). Os macroporos, no campo dos biomateriais, são poros com um diâmetro superior a 50µm, que permitem o crescimento ósseo (Karageorgiou and Kaplan 2005, Böhner, Galea et al. 2012). Revendo a bibliografia disponível, vários estudos apontam diferentes diâmetros para o tamanho dos macroporos dos biomateriais. Nos primeiros trabalhos, onde se estudou o efeito da porosidade dos biomateriais na osteogênese, Hulbert e colaboradores concluíram que o tamanho mínimo dos poros para haver crescimento ósseo devia estar entre 75 a 100µm (Hulbert, Young et al. 1970). Estudos mais recentes mencionam que o crescimento ósseo é menor em cerâmicos com poros menores que 80 a 140µm (Galois and Mainard 2004). Um ponto importante no tamanho do macroporo é ter dimensões suficientes para permitir que as células possam migrar para o seu interior, sendo esse tamanho apontado como superior a 100µm (Karageorgiou and Kaplan 2005), mas num estudo com HA e β FTC Lu, Flautre et al. (1999) verificaram que os *osteoblastos in vitro* penetravam em poros de 20µm, mas que *in vivo*, apesar dos osteoblastos ter a capacidade de penetrar esses poros, só se formava tecido cartilaginoso, concluindo que para a formação de osso, os poros tinham que ter obrigatoriamente dimensões superiores a 50µm (Lu, Flautre et al. 1999). Considerando as dimensões dos macroporos, também se deve ter em conta a capacidade da invasão vascular com a proliferação de capilares (capacidade de angiogênese), para haver uma boa oxigenação e formação direta de tecido ósseo, pois situações de hipoxia levam à formação osteocondral e só posteriormente à ossificação. Sendo assim, os macroporos deverão ter dimensões que permitam esta invasão vascular, estando indicado para esse

efeito dimensões superiores a 300µm (Tsuruga, Takita et al. 1997, Kuboki, Jin et al. 2001, Karageorgiou and Kaplan 2005). Também existe um limiar a partir do qual o aumento do tamanho dos poros é prejudicial à regeneração óssea, com problemas na osteointegração e contacto do osso com biomaterial (Götz, Müller et al. 2004), podendo, também, os diâmetros demasiado elevadas dificultarem o preenchimento dos poros por células (Le Huec, Clement et al. 2000). Tsuruga e colaboradores verificaram que dimensões de poros superiores e inferiores a 300-400 µm produziam menos osso, pondo a hipótese que os poros de 300 a 400 µm se adaptavam melhor ao tamanho dos osteomas (200 a 300 µm) (Tsuruga, Takita et al. 1997).

O tamanho da porosidade também deverá ter em conta a taxa de degradação do biomaterial, pois porosidades elevadas, em materiais muito degradáveis, poderão levar a uma absorção rápida e consequente perda de função do implante antes de crescimento de osso novo (Karageorgiou and Kaplan 2005). Devido à reabsorção rápida, o limiar do diâmetro dos poros, a partir do qual se verifica crescimento ósseo, em materiais reabsorvíveis, poderá ser menor, pois ao longo do tempo com o processo de reabsorção a decorrer, o diâmetro desses poros vai aumentando. Num trabalho onde foi comparado o comportamento da HA e do β FTC, com porosidades de 45-80µm, 80-140µm, 140-200µm, e 200-250µm em vários tempos de implantação (2 semanas, 1 mês, 2 meses, 4 meses, 6 meses e 12 meses), foi verificado que, mesmo no material com poros de 45-80µm houve invasão óssea, apesar de ter sido retardada em relação às porosidades maiores. Nos implantes de HA com poros de tamanho 45-80µm, apresentaram, em todos os tempos de implantação, uma menor quantidade de osso, com diferenças com significado estatístico, quando comparados com os implantes com porosidades maiores. Este comportamento já não se verificou no caso da β -FTC, que só nos tempos de implantação inferiores a 4 meses é que apresentaram menos osso que os restantes grupos experimentais, sendo a quantidade de osso semelhante nos restantes tempos de implantação testados. Através destes resultados, os autores concluíram que a composição química influencia a capacidade do osso invadir os macroporos e que substâncias com taxas de reabsorção maiores, como o caso da β -FTC em relação a HA, permitem, a partir de um certo tempo decorrido, a criação de espaços maiores para a invasão de osso novo (Galois and Mainard 2004).

Além da macroporosidade ter um impacto fundamental na osteogénese, a microporosidade é, sem dúvida, outro fator relevante que se tem que ter em conta na produção de enxertos ósseos sintéticos (Yuan, Kurashina et al. 1999, Bignon, Chouteau et al. 2003, Annaz, Hing et al. 2004, Karageorgiou and Kaplan 2005).

Microporos são definidos como poros do biomaterial com tamanhos inferiores a 10µm. A presença e o incremento destes microporos fazem com que a área de superfície de contacto do material aumente (Yuan, Kurashina et al. 1999). Quando se recorre à utilização de biomateriais, no processo de regeneração óssea, a atividade das células precursoras está dependente da ancoragem à superfície do biomaterial, para se diferenciarem, proliferarem e iniciarem a produção de matriz óssea mineralizada (Osborn 1991, Lin, Su et al. 1997, Okumura, Ohgushi et al. 1997, Yang, Yuan et al. 1997). Outra etapa importante é a presença e absorção, pelo biomaterial, de proteínas, nomeadamente proteínas morfogénicas do osso (BMPs), indispensáveis para o início da osteogénese. Esta indução da osteogénese, por parte das BMPs, está depende do tipo e da concentração destas BMPs no microambiente, existindo um limiar, a partir do qual as BMPs começam a estimular a produção de osso (Wozney, Rosen et al. 1988, Wang, Rosen et al. 1990, Fleet, Cashman et al. 1996, Riley, Lane et al. 1996, Volek-Smith and Urist 1996, Carreira, Lojudice et al. 2014). Como foi referido, os microporos aumentam a área de contacto do biomaterial, permitindo assim que mais células possam ancorar, proliferar e diferenciar-se na superfície do biomaterial. Está igualmente descrito que uma superfície rugosa, devido à presença dos microporos, facilita o processo de adesão e expansão celular sobre o biomaterial (Kawai, Niwa et al. 1997, Lampin, Warocquier et al. 1997, Lin, Su et al. 1997). Em áreas de superfície maiores, a adsorção de proteínas também vai aumentar, conseguindo-se assim, atingir o limiar de quantidade de BMPs que estimula a produção óssea, mais cedo. Em áreas de superfície pequenas, a concentração de BMPs adsorvidas poderá nunca ser suficiente para o estímulo da produção óssea, mesmo em tempos de implantação muito alargados. A microestrutura com a presença de microporos aumenta e facilita as trocas iónicas e a formação de apatites carbonadas com os processos de dissolução e reprecipitação, favorecendo e fortalecendo as ligações diretas entre o osso e o biomaterial implantado (Daculsi, LeGeros et al.

1990, Hing 2005). A interconexão dos microporos permite ainda criar um microambiente favorável à diferenciação celular e deposição de matriz óssea (Kawai, Niwa et al. 1997), possibilitando a troca e circulação de fluídos e iões, com um incremento na comunicação e interação entre as células (Kawai, Niwa et al. 1997).

Outro parâmetro importante, relacionado com a macroporosidade e a microporosidade, refere-se à porosidade total. O crescimento ósseo é promovido com o aumento da porosidade total, mas este incremento compromete a resistência mecânica (Gauthier, Bouler et al. 1998). Para satisfazer estas duas condicionantes, vários estudos apontam para que porosidade total deve estar entre os 40% e os 60% (Passuti, Daculsi et al. 1989, Grundel, Chapman et al. 1991, Gauthier, Bouler et al. 1998, Barralet, Grover et al. 2002, Hing 2005, Karageorgiou and Kaplan 2005).

Tendo em conta que o crescimento ósseo tem um padrão circular, a forma esférica dos grânulos poderá ser mais favorável ao crescimento e aposição óssea, além disso, material com formas poligonais, apresenta arestas aguçadas que poderão dificultar a aposição de osso. A mobilidade das partículas afeta negativamente o desenvolvimento vascular e consequentemente o crescimento ósseo (Reis, Borges et al. 2010), sendo esta situação agravada no caso de partículas com bordos aguçados, pois este tipo de superfície está mais propenso a traumatizar o tecido envolvente.

Modelos animais utilizados na testagem *in vivo* de biomateriais:

Previamente à utilização clínica dos biomateriais, deverão ser realizados vários estudos onde se avalia a segurança da sua utilização (os biomateriais não deverão ser tóxicos nem carcinogénicos), a sua citocompatibilidade e a sua histocompatibilidade. Além da caracterização físico-química é importante assegurar a biocompatibilidade do material e a sua eficácia na osteocondução, osteointegração, osteoindução e na adesão celular (Pearce, Richards et al. 2007). Para isto são necessários realizar teste rigorosos tanto *in vitro*, como *in vivo*.

Tendo em conta o bem-estar animal, elemento fundamental do desenvolvimento da ciência atual, a experimentação animal deverá ser reduzida ao máximo, mas infelizmente os testes *in vitro* não replicam por completo o comportamento e as reações dos tecidos vivos, sendo sempre necessária uma pré-avaliação dos biomateriais em modelos animais, se possível, o mais próximo possível do Homem em termos de fisiologia e anatomia. Mesmo assim, o estudo preliminar *in vitro* é indispensável, não só para avaliar as características físicas e químicas do material, bem como a sua citocompatibilidade. Este processo evita a aplicação de biomateriais em modelos animais, que à partida não vão obter resultados positivos (Martini, Fini et al. 2001, Pearce, Richards et al. 2007).

Na escolha do modelo animal, a variabilidade individual deve ser reduzida ao máximo, controlando o grupo de animais quanto ao sexo, idade, peso, raça e condições ambientais. Também tem que se ter em conta, o conhecimento do comportamento biológico da espécie utilizada como modelo, bem como a utilização dessa espécie em trabalhos de outros autores, para assim se poderem comparar resultados. Para se poder extrapolar e prever o comportamento nos humanos, é importante que o modelo apresente analogia com os humanos. Outros pontos a considerar são o custo de aquisição, manutenção e facilidade de manipulação dos animais. A aceitação pela sociedade e as consequências ecológicas da utilização do modelo animal escolhido também não podem ser esquecidos (Davidson, Lindsey et al. 1987, Martini, Fini et al. 2001, Pearce, Richards et al. 2007, Barradas, Yuan et al. 2011).

Os primatas poderão ser considerados um excelente modelo animal, devido às suas semelhanças com os humanos, mas eticamente a utilização desta espécie não é muito bem aceite pela sociedade. Além disso este é um modelo muito caro que requer controlos rigorosos e tem um grande potencial para transmissão de zoonoses. Os canídeos são uma espécie bastante usada na testagem de substitutos ósseos, mas existem pressões emocionais e éticas para a restrição do uso de animais de companhia em experimentação animal, sendo a utilização de animais de laboratório e de espécies pecuárias como os porcos, as cabras e as ovelhas, mais bem aceite pela sociedade (Pearce, Richards et al. 2007).

Os animais de laboratório são um modelo animal utilizado com bastante frequência em estudos ortopédicos, devido ao seu baixo custo, à fácil

manutenção e manipulação e aos vários estudos existentes, com um bom conhecimento do comportamento biológico deste modelo. No entanto este modelo apresenta grandes diferenças em relação aos humanos, como por exemplo a carga suportada pelo osso. Além disso as pequenas dimensões destas espécies limitam o tamanho e o número de implantes que podem ser utilizados. Apesar dos suínos apresentarem algumas analogias fisiológicas com os humanos, existem problemas relacionados com o tamanho e peso dos animais adultos, bem como a baixa tolerância à manipulação (Newman, Turner et al. 1995, Martini, Fini et al. 2001, Pearce, Richards et al. 2007).

A utilização dos ovinos como modelo animal, para situações ortopédicas, tem aumentado de forma acentuada, nas últimas décadas (Martini, Fini et al. 2001). Esta espécie apresenta diversas vantagens, sendo uma espécie de produção zootécnica e por isso a sua utilização em experimentação animal é mais bem aceite pela sociedade. O peso de um ovino adulto é comparável ao de um humano, refletindo uma carga mecânica semelhante aos humanos; as dimensões dos ossos são apropriadas para a utilização de biomateriais e próteses com tamanhos semelhantes àsquelas utilizadas em humanos. Permite ainda a aplicação de diversas amostras e a realização de múltiplos procedimentos cirúrgicos no mesmo animal; para além do facto de as ovelhas serem animais bastantes dóceis e de fácil manipulação e o seu custo de aquisição e manutenção ser acessível. Embora os caprinos apresentem as mesmas vantagens dos ovinos, a manipulação e confinamento das cabras é mais problemático (Newman, Turner et al. 1995, Martini, Fini et al. 2001, Pearce, Richards et al. 2007).

Análises histomorfométricas:

Nos modelos animais, para realizar a avaliação do comportamento de um enxerto ósseo, devem ser realizadas análises histomorfométricas, com o objetivo de avaliar a *performance* do biomaterial, estimando o grau de osteointegração, osteocondução e a taxa de reabsorção, de uma forma precisa e reprodutível. Para cumprir este objetivo devem ser calculadas as áreas preenchidas do defeito, as áreas das diferentes frações (osso, tecido conjuntivo, lacunas,

biomaterial), perímetros de contacto entre osso e biomaterial e a quantidade de osso novo e de biomaterial (Hallman, Cederlund et al. 2001, Norton, Odell et al. 2003, Somanathan and Simunek 2006). Vários métodos estão descritos para realizar estas análises histomorfométricas, alguns são muito trabalhosos e morosos, como por exemplo, a delimitação das diversas frações ou a utilização de grelhas de contagem (Gundersen, Bendtsen et al. 1988, Lopes, Santos et al. 2001, Mandarin-de-Lacerda 2003, Norton, Odell et al. 2003, Cortez, Silva et al. 2011). Outra técnica mais simples e menos trabalhosa e morosa é a utilização da segmentação da imagem computadorizada (*Thresholding*) (Van der Pol, Mathieu et al. 2010). A segmentação da imagem consiste na separação da imagem agrupando os pixéis que têm o mesmo atributo, podendo assim as frações em estudo serem selecionadas de acordo com a sua escala de cinza. Normalmente o método de *Threshold* requer a análise do histograma da imagem, que representa graficamente a frequência de cada pixel, no eixo vertical e no eixo horizontal, o valor de *Threshold*. O valor observado varia desde 0, que corresponde aos pixéis pretos, até 255, que corresponde aos pixéis brancos, os outros valores intermédios são os diferentes níveis de cinzento (**Figura 3**). Quando as imagens têm um bom contraste, o histograma vai ter picos de frequência distintos, sendo possível e fácil aplicar o método de *Threshold*, para dividir a imagem e selecionar as diferentes áreas de interesse. Sempre que o *Threshold* é aplicado, os pixéis são classificados em 2 categorias, uma onde os valores dos pixéis estão dentro do nível de *Threshold* selecionado e outro onde os pixéis estão fora deste nível, criando-se uma imagem binária. A escolha do valor de *Thredshold*, para selecionar a fração pretendida, poderá ser feita visualmente, por um operador, regulando e procurando o valor de corte que apresente resultados satisfatórios. Outra alternativa é criar um valor fixo independente do operador e que apresente sempre bons resultados, como por exemplo no caso de imagens com distintos picos do histograma, optar-se por escolher o valor de *Threshold* entre dois picos (na vala do histograma) (**Figura 4**) (Efford 2000, Burger and Burge 2009).

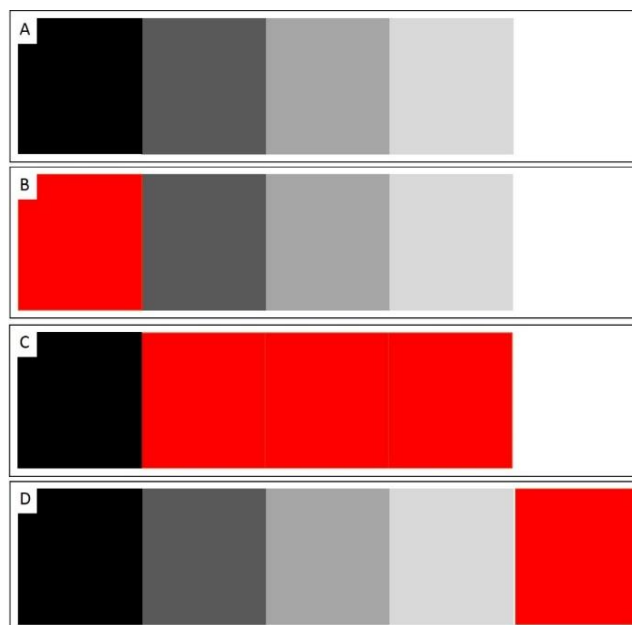


Figura 3: No método de *Threshold* a fração pretendida é selecionada de acordo com a escala de cinzento (A), onde o valor 0 representa o preto (B), o 255 o branco (D), e os valores compreendidos entre 2 e 254, as várias tonalidades de cinzento (C).

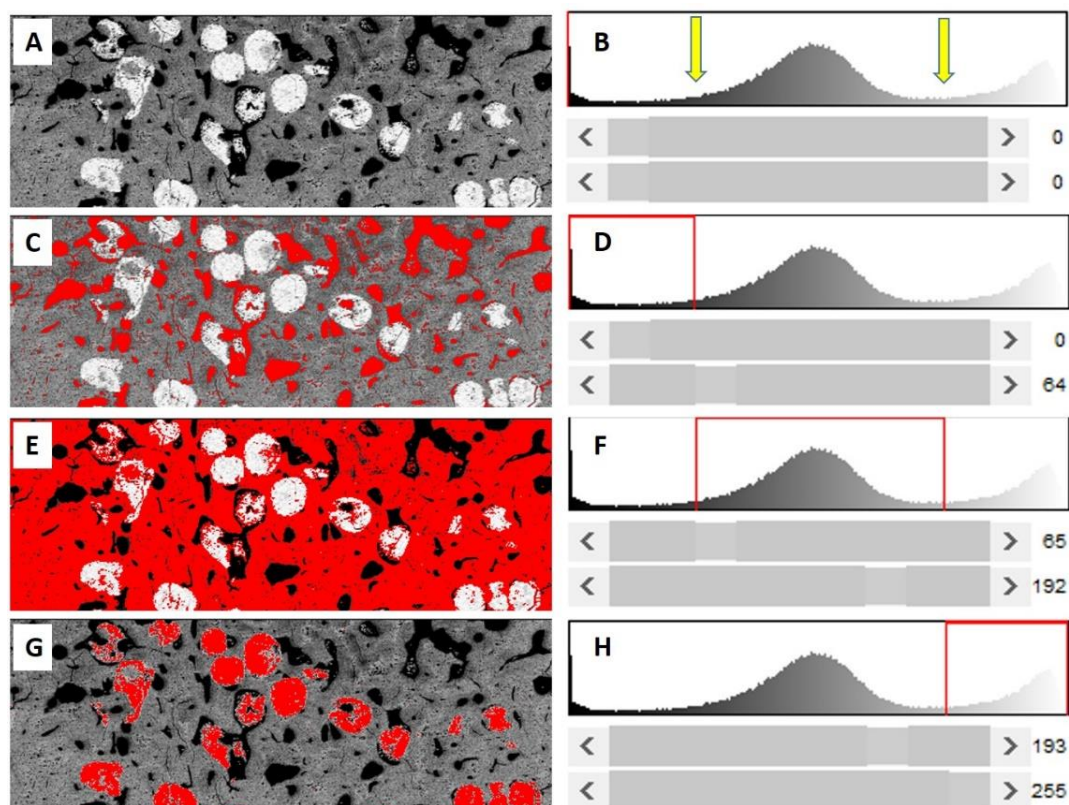


Figura 4: Análise de *Threshold* de uma imagem de SEM de tecido ósseo onde foi implantado Bonelike® (A, C, E e G). A segmentação da imagem foi feita com auxílio do histograma (B, D, F e G), com a zona de corte na vala do histograma (seta amarela e linhas vermelhas). A – imagem por analisar, C – seleção das lacunas, E – seleção do osso, G – seleção do biomaterial.

Enxertos ósseos em Medicina Veterinária:

A utilização de enxertos ósseos sintéticos tem vindo a aumentar em Medicina Veterinária, mas mesmo assim a sua aplicação é substancialmente menor em comparação com a aplicação em Medicina Humana. Isto deve-se principalmente aos custos envolvidos na utilização de biomaterias. Mas com o desenvolvimento da sociedade e consequente atenção dada aos animais de companhia, verificou-se um aumento da disponibilidade dos proprietários em investir no tratamento dos seus animais recorrendo a biomateriais. Além do valor sentimental de certos animais, não é de desprezar o crescimento da valorização económica de certo tipo de animais. Por exemplo no caso dos equinos, a acompanhar algumas modalidades equestres, gera-se uma indústria que movimenta avultadas quantias financeiras, atingindo o preço de alguns cavalos valores astronómicos. Com esta mudança de mentalidades e de condicionalismos, a utilização dos biomateriais em animais de companhia e de interesse zootécnico, deixa de se restringir aos ensaios pré-clínicos realizados antes da sua aplicação em humanos, e passa a fazer parte dos procedimentos disponíveis aos clínicos veterinários. Estas técnicas podem ser utilizadas, em inúmeras situações clínicas de Medicina Veterinária, em que haja um defeito ósseo, como por exemplo algumas fraturas como as fraturas cominutivas, preenchimento de quistos ósseos e artrodeses. (Schwarz, Schlag et al. 1991, Duval, Chambers et al. 1995, KERWIN, LEWIS et al. 1996, Dowdle, Spotswood et al. 2003, Kim, Choi et al. 2005, Vertenten, Gasthuys et al. 2010).

Artrodeses em equinos:

A artrodese é um procedimento cirúrgico que tem como finalidade a promoção da imobilização e fusão permanente duma articulação. Este procedimento é utilizado, tanto em Medicina Veterinária como em Medicina Humana, tendo como objetivo diminuir a dor existente e/ou deformidades presentes em algumas patologias (Lesser 2002, Zubrod and Schneider 2005,

Schoenhaus, Lam et al. 2009, Sorrel Langley-Hobbs M.A. 2010). Este procedimento está indicado para o tratamento da doença degenerativa articular (artrose), alguns casos de luxações, lesões severas de ligamentos, e alguns casos de fraturas articulares (Lesser 2002, Zubrod and Schneider 2005, Schoenhaus, Lam et al. 2009, Sorrel Langley-Hobbs M.A. 2010).

Normalmente recorre-se a este tipo de cirurgia quando não há uma resposta a tratamentos médicos convencionais, servindo assim, este procedimento cirúrgico, para aliviar a dor e a disfunção da articulação (Johnson 1995, Lesser 2002, Zubrod and Schneider 2005). No caso específico dos cavalos, o principal objetivo é permitir que os animais recuperem e retomem a sua atividade desportiva. No entanto, quando isto não é possível, este tipo de cirurgia continua a ser extremamente útil para diminuir a dor e aumentar o conforto do animal, assim como o uso do membro afetado, prevenindo assim, o aparecimento de lesões de sobrecarga no membro contralateral. As articulações em que se consegue uma recuperação total são as interfalângicas proximais, intertársicas distais e tarso metatársicas, pois estas articulações apresentam naturalmente pouco movimento, e a sua imobilização e fusão permanente não afeta a locomoção normal (Atayde and Mendes 2004, Zubrod and Schneider 2005, Zubrod, Schneider et al. 2005, Sorrel Langley-Hobbs M.A. 2010).

Na evolução natural de uma artrose, verificam-se processos degenerativos, que se iniciam na cartilagem articular. A degradação da cartilagem diminui a absorção de forças por parte desta estrutura, sobrecarregando o osso subcondral. Esta sobrecarga leva a formação de esclerose no osso subcondral, perdendo estas capacidades de absorção de choques, sobrecarregando assim a cartilagem articular. Este ciclo degenerativo culmina com o desaparecimento da cartilagem, ficando as extremidades ósseas em contacto, formando-se assim uma anquilose. Com a formação da anquilose, a situação clínica do animal melhora, devido ao desaparecimento da dor associada ao movimento da articulação. O grande problema prende-se com o facto, de que, este processo natural da formação da anquilose poder demorar bastante tempo e nalgumas situações não ser efetivo, continuando o cavalo com dor e desconforto. Nesse sentido, a artrodese cirúrgica deve ser uma opção para o tratamento desta patologia (Atayde and Mendes 2004, Zubrod and Schneider 2005).

A técnica de artrodese consiste em desbridar a cartilagem articular, para que haja um contacto entre os dois extremos ósseos e assim a articulação se funda, sendo também necessário a realização do alinhamento e estabilização da articulação na posição fisiológica de apoio. Vários métodos de fixação estão descritos para se conseguir a estabilização e alinhamento da articulação. A escolha do método a usar depende do tipo de articulação envolvida (Atayde and Mendes 2004, Zubrod and Schneider 2005). Está descrito o uso de autoenxertos para acelerar o processo, melhorar o contacto e preencher pequenas lacunas entre os dois extremos ósseos (Johnson 1995, Lesser 2002). A eventual utilização do Bonelike® poderá apresentar vantagens em relação a estes autoenxertos.

Capítulo 2

Objetivos

2 – Objetivos:

Na primeira fase deste trabalho experimental conducente ao grau de Doutor, pretendeu-se descrever e validar um modelo animal para estudar biomateriais na regeneração óssea, que permitisse comparar diferentes composições de materiais, com resultados fiáveis, em períodos de experimentação curtos e com a possibilidade de restringir o número de animais, mantendo a viabilidade da realização de testes com significado estatístico. No modelo desenvolvido foi possível ainda descrever detalhadamente o processo de regeneração óssea nos defeitos circulares com 5 mm de diâmetro criados em ambos os fêmures de ovelhas. Outro objetivo a atingir nesta tese foi o desenvolvimento de um método reprodutível, fiável, rápido e objetivo, para a realização da análise histomorfométrica do tecido ósseo após determinado período de implantação, no sentido de analisar de forma qualitativa mas também quantitativa, a regeneração óssea. Depois de desenvolvido o modelo animal e o método de análise histomorfométrica do tecido ósseo regenerado, pretendeu-se ainda estudar uma nova formulação de Bonelike® com formato esférico (*pellets*), com duas granulometrias distintas, nomeadamente 250-500µm e 500-1000µm. Foi realizada a sua caracterização física e química e foi avaliada a sua *performance* e comportamento no modelo animal ovino desenvolvido e descrito nesta tese. Outro objetivo a alcançar foi a avaliação da influência da variação do formato e tamanho dos grânulos de Bonelike® na regeneração óssea através de estudos *in vivo*. Nestes estudos pretende-se comparar o comportamento *in vivo*, de diferentes formulações e tamanhos de formas granulares de Bonelike®, para assim avaliar a influência da forma física, estrutura e microestrutura dos biomateriais na regeneração óssea. Por último pretendeu-se aplicar o Bonelike® esférico em casos clínicos de Medicina Veterinária, nomeadamente em artrodeses de cavalos.

Capítulo 3

Modelo ovino para testagem de biomateriais

3 – Modelo ovino para testagem de biomateriais

Neste capítulo apresentam-se os trabalhos onde se desenvolveu e validou o modelo animal proposto. Descreve-se detalhadamente a regeneração óssea em defeitos circulares com 5 mm de diâmetro criados em ambos os fêmures dos animais de experimentação. Nestes trabalhos, também se desenvolveu e validou um novo método de análise histomorfométrica, de segmentação de imagem (Método *Threshold*). Este método permitiu realizar a avaliação do comportamento dos biomateriais na regeneração do tecido ósseo de forma estandardizada e reproduzível.

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A new sheep model with computing automatized analysis to evaluate the *in vivo* biomaterial's behavior on bone tissue regeneration.

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A new sheep model with computing automatized analysis to evaluate the *in vivo* biomaterial's behavior on bone tissue regeneration.

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Abstract

Presently, several bone graft substitutes are being developed or already available for clinical use. However, the limited number of clinical and *in vivo* trials for direct comparison between these products may complicate this choice. One of the main reasons for this scarcity it is the use of models that do not readily allow the direct comparison of multiple bone graft substitutes, due especially to the small number of implantation sites. Although sheep cancellous bone models are now well established for these purposes, the limited availability of cancellous bone makes it difficult to find multiple comparable sites within a same animal. These limitations can be overcome by the monocortical model here proposed as it consists in 5 to 6 holes (5 mm Ø), in the femoral diaphysis, with similar bone structure, overlying soft tissue and loading pattern for all defects. Associated to this model, it is also described a fast histomorphometric analysis method using a computer image segmentation test (*Threshold method*) to assess bone regeneration parameters. The information compiled through the experimental use of 45 sheep in several studies allowed determining that this ovine model has the potential to demonstrate differences in bone-forming *performance* between various scaffolds. Additionally, the described histomorphometric method is fast, accurate and reproducible.

Keywords: bone regeneration, animal model, sheep, biomaterials, histomorphometry, Threshold method

1 – Introduction

In the last decades, several biomaterials have been developed as bone substitutes but before the clinical trials, several studies must be performed to evaluate safety, biocompatibility, osteoconduction, osteointegration and osteoinduction (1). Unfortunately, as current *in vitro* models do not replicate the tissue response of a live animal to a bone substitute (1-3), several animal models have been used by different research groups. The selection of this model must follow some important concerns: the group of animals used must be controlled for sex, age, weight and strain (when possible); demonstration of analogy with humans; background knowledge of biological properties; cost of acquisition and maintenance of the animals; availability; handling and housing easily; ethical implications; and the ecological consequences (1, 2, 4, 5). The sheep model has gradually increased its importance in the last decades as a model for bone regeneration studies (2). First, as a food animal species is ethically more acceptable; the body weight makes the biomechanical and loading processes more similar to humans. Additionally sheep are very docile, easy to handle and house and the cost of purchase and housing is not too high, permitting large scale studies (1, 2, 6). Several bones have been used to study biomaterials and implants using the sheep model (7-15). Some of these models require 2 or more incision sites and there is a restrict number of samples that can be implanted per animal. In these specific cases, care must be taken with model planning, considering the differences that may exist in bone structure, bone loading and amount of overlying soft tissue, since these factors can influence the healing process (7, 16-19).

Cancellous bone models are now well established for the assessment of new bone substitutes, but the limited availability makes it difficult to find multiple comparable sites within a same animal (18), which may compromise the comparison of the *in vivo* performance of different biomaterials (20). With cortical bone models it is easier to find multiple sites to implant the studied bone grafts, allowing serial sampling in the same animal, which is in accordance with the 3R's policy, designing the experience towards maximum information with minimum animal use (21).

In order to evaluate the behavior of biomaterials in bone tissue regeneration, a histomorphometric analysis allows an accurate, reproducible and quantitative way for estimating the osteointegration, osteoconduction and degree of biomaterial degradation. In this sense, the area of the different fractions (bone, conjunctive tissue, lacunae, biomaterial), and contact perimeters between material and newly formed bone should be calculated (22-25).

Several methods can be used to perform histomorfometric measurements, but the ones that limit the contour of the intended fraction are laborious (23, 26-29). Another less laborious alternative to calculate a fraction area is the use of image segmentation (*Threshold* method) (16, 25), which consists in the image separation, by grouping together pixels that have the same attribute. This method is particularly straightforward on scanning electron microscopy (SEM) images due to the marked contrast between new bone, empty spaces and biomaterials. The *threshold* processing can be done by visualization, varying the threshold selection until acceptable results are achieved. Another possibility is to find a fixed threshold value that will give good and reliable results (30, 31).

The work described here was carried out based on the gathered experience of previous studies of our research group concerning the performance of several biomaterials on bone regeneration (25, 29) and describes the sheep model used. The objective of this study is to demonstrate the feasibility of an *in vivo* model to detect significant differences, for parameters related to bone regeneration, between various combinations of scaffolds through the quantitative assessment of results in order to provide clinically relevant outcome criteria.

2 – Materials and Methods

2.1 – *In vivo* experiments:

Forty five healthy skeletally mature Merino sheep, with an average weight of 50 kg, were used as experimental models. All procedures were performed with the approval of the veterinary authorities of Portugal in accordance with the European Communities Council Directive 86/609/EEC.

2.1.1 – Surgical procedure:

Surgical technique

Surgeries were conducted under general anesthesia. The skin incision was made laterally above the diaphysis of the femur along its entire cranial edge (**Fig. 1A and 1B**). The *fascia lata* was incised along the cranial border of the biceps femoral muscle. To expose the femur diaphysis, the *vastus laterallis* muscle was retracted cranially and the biceps femoral retracted caudally (**Fig. 1C and 1D**).

Defect creation

In the lateral diaphysis of each sheep's femur, a row of five holes with a 5.0 mm diameter was drilled through the cortex and into the medulla, using a microburr continuously flushed with a normal sterile saline solution to minimize thermal damage and to remove any residual bone (**Fig. 2A**). A minimal distance of 1 cm was kept between drill holes to reduce the risk of fracture. In this animal model, the defects were filled with the tested biomaterial or left empty as a negative control. The biomaterial used in a large number of *in vivo* testing was Bonelike® (modified hydroxyapatite matrix with tricalcium phosphate β - and α - secondary phases (25, 32)), with two different formats, polygonal granules (29) and spherical particles (25), and two ranges of granule sizes (250-500 μ m, and 500-1000 μ m). Bonelike® was applied premixed with autologous peripheral blood or a vehicle, and the complete filling of the defects was ensured (**Fig. 2B and 2C**).

Post-surgical procedures

After recovering from anesthesia, animals were transferred either to a straw yard, together with other animals (Group 1, N = 5 animals), or into an individual cage (Group 2, N = 40 animals) (**Fig. 2D**). All animals were subjected to post-surgical antibiotherapy and analgesia. Lateral-medial and dorso-plantar X-ray projections were taken for the healing process follow up. An intravenous injection of 20% pentobarbital sodium solution was used to sacrifice the animals at different implanting times (days after the surgery). Both femurs of each animal were dissected and all soft tissue was stripped from the collected bones in order to prepare the histological analysis of the samples.

2.2 – Histological and Histomorphometric analysis:

After bone dissection and removal of soft tissue, two X-ray projections (lateral-medial and dorso-plantar) were performed. In order to clearly identify the bone defects, three techniques were applied: 1) direct visualization and macroscopic evaluation; 2) use of X-ray and measuring the location of the defect; and 3) cutting out the femoral head, removing the medulla to search for internal prominences which correspond to the defect. After locating the bone defect, two transversal lines were drawn 5 mm proximal and distal to the defect, to serve as cutting guides. The segments containing the defects between the drawn lines were cut with a saw perpendicularly to the long femur axis, and immersed in neutral formaldehyde (10%). Samples were afterwards maintained in a neutral formaldehyde solution (10%) for 7 days; the segments were dehydrated in an increased

percentage of alcohol solutions, and embedded in a methylmethacrylate resin. Thin sections with 150 μm were cut perpendicularly to the femur axis with a diamond blade microtome and hand-ground to approximately 70–80 μm . Sections were then stained with Solochrome Cyanine R and Hematoxylin & Eosin for histological examination with a Nikon light microscope (Eclipse E600, Nikon, Tokyo, Japan), equipped with a calibrated digital camera (Nikon DS-5M-L1 Digital Sight Camera System, Nikon). Unstained slides were used to perform scan electron microscopy (SEM) analyzes (FEI Quanta 400 FEG ESEM) operating in high vacuum mode at an accelerating voltage of 15 kV SEM. In this work 29 empty holes ($N = 29$) with different implantation times: 30 days ($N=13$); 60 days ($N=4$); 120 days ($N=7$) and 180 days ($N=5$) were analyzed, to characterize and report the bone healing process of the defects in the developed animal model. The image was divided into 5 fractions (**Fig. 3**), namely: 1) the defect area was defined as a quadrangular area with its vertices intercepting the defect wall, two periosteum and two medullar edges; 2) the medullar callus which was the zone with new bone formation, underneath the defect area; 3) the cortical callus which was the zone with new bone. formation, over the defect area; 4) the endosteum callus on the defect neighboring zone, with 1000 μm width; and 5) the periosteum callus on the defect neighboring zone, with 1000 μm width.

The histological images were analyzed to evaluate the cellular reaction and the quality of new bone formation, permitting the characterization of the events of bone formation and remodeling process during the healing process.

Threshold analysis

The *Threshold* method was used to perform the histomorphometric analysis. With this method, the image can be separated according to the grey level of each region of interest by analyzing the image histogram. The histogram describes the frequency of each pixel and it is represented by a graphic, where the vertical axis is the frequency of each pixel and the horizontal axis is the threshold value. The threshold value varies between 0 (the black pixels) and 255 (the white pixels); the other values are different grey levels. When the image has good contrast, the histogram presents distinct peaks of frequency and it is possible to divide the image in different areas of interest. When the *Threshold method* is applied, the pixels are classified into two categories: one where the pixel values fall into the threshold level and another where they fall outside the threshold level, and therefore creating a binary image.

For the *Threshold analysis*, the images were converted into 8-bit grey scale (**Movie 1**). SEM images are already obtained in a grey scale, which is advantageous to this analysis method. The *Image J* software was used to perform the *Threshold* analysis (33), applying image adjustment with the *Threshold* tool (Image / Adjust / Threshold) (**Movie 2**). With the aid of the obtained histogram, separation and measurement of the different areas of the studied fractions (lacunae, bone and biomaterial) were performed (**Fig. 4**). A minimum and maximum threshold value was defined to separate these fractions and the cutting areas of the different fractions were also calculated using the obtained histogram information. To select the lacunae (black pixels), the threshold started with 0 and ended halfway between 0 and the medium peak of the histogram (**Fig. 4A, 5A and 5B; Movie 3**). To select the bone (grey pixels), the threshold started one point after the maximum threshold value of the previous fraction and ended halfway between the medium peak of the histogram and the maximum threshold value (255) (**Fig. 4B and 5C; Movie 4**). Finally, to select the biomaterial (white pixels), the threshold started one point after the maximum threshold value of the previous fraction and ended in 255 (**Fig. 4C and 5D; Movie 5**). Also, the following quantifications were performed: the mean size of the defect, for all the samples included in this study, and within the different experimental groups (considering different implantation times: 30 days, 60 days, , and 180 days), the mean percentage of defects with bone contact between edges of the defect; the mean percentage of unfilled area of the defect; the mean percentage of medullar callus in relation to the defect (medullar callus area / defect area x 100); the mean percentage of cortical callus in relation to the defect (cortical callus area / defect area x 100); the mean percentage of endosteum callus on a neighboring zone of the defect, with a 1000 μm width, in relation to the defect (endosteum callus area / defect area x 100); the mean percentage of periosteum callus on a neighboring zone of the defect, with a 1000 μm width, in relation to the defect (pereosteum callus area / defect area x 100); and the mean area fraction occupied by lacunae and new bone, on the defect area (in this parameter, for the implantation time of 30 days, the group was split in two groups depending on having more or less than 50% of unfilled area). Data were presented as means and stander deviations (SD). To compare mean percentage on different implantations times, the one way ANOVA analyze with LSD post hoc test was used.

3 – Results

The surgical procedure was simple, fast and well tolerated by the animals. The animals could fully weight-bearing on the first post-surgical day. No post-surgical infections were observed and the surgical skin incision healed without any complication. The individual cage confinement was well tolerated by all sheep, showing no signs of discomfort or stress. During the healing period after the surgery, femur fractures occurred in 2 animals of group A (40% of fractures, 2 out of 5 animals in total) and in 2 animals of group B (5% of fractures, 2 out of 40 animals in total). On the radiographs taken at the first post-surgical day, the empty holes (control) were observed as radiolucent circles and the holes with biomaterial, as radiopaque holes. As implantation time increased, the visualization of empty holes on X-rays became more difficult. In some cases with implantation times longer than 90 days, empty holes could not be differentiated from the remaining bone (**Fig. 6C, 6D, 6E and 6F**). On a dorso-plantar X-ray projection, it could be observed that some granules of biomaterial were displaced into the medullar space beside the defect, and that new bone had formed around the biomaterial (**Fig. 6B, 6D and 6F**). On post mortem femur preparation at 30 days implantation time, all the defects were easily identified, but with implantations times longer than 60 days, defects visualization was difficult (**Fig. 7**).

By histological analysis of controls (empty holes), it was observed, at implantation times of 30 and 60 days, absorption lacunae on un-operated bone, on edges of the defect (**Fig. 8A and Fig. 8B**). This feature was more prevalent on samples that showed better osteo-regeneration. At longer implantation times, this feature disappeared (**Fig. 8C and Fig. 8D**). Thirty days after implantation, the majority of new bone presented a trabecular pattern, with clustered osteocytes lacunae surrounded by small quantities of bone matrix, and in a random orientation like the woven bone (**Fig. 8A and Fig. 8E**). At implantation time of 60 days, the new bone was molded to a more organized structure, with a lamellar conformation at the right angles to the longitudinal axis of the bone; also, this new bone began to adopt a more compact pattern (**Fig. 8B and Fig. 8F**). At implantation time of 120 days, the molding process progressed and the new bone showed a lamellar structure at the right angles to the longitudinal axis, the bone matrix increased, with osteocyte lacunae surrounded by more bone and increased distance between them (**Fig. 8C and Fig. 8G**). At implantation time of 180 days, on some locations of certain samples, the beginning of the remodeling process was observed, with bone adopting the lamellar pattern, at normal orientation, parallel to the longitudinal axis. The amount of new bone

surrounding each osteocyte lacuna was the same observed on a normal healed bone (**Fig. 8D and Fig. 8H**).

The mean size of the defect on histological samples was 3.87 ± 0.6 mm width and 2.36 ± 0.32 mm height. At 30 days of implantation time and for empty holes, only 38.50% of animals showed bone bridging between the edges of the defect. For the other implantation times, this was a constant observation. The mean size of unfilled area decreased as the implantation time increased, so the mean size of unfilled area at 120 days and 180 days of implantation time was significantly smaller when compared to the mean size of unfilled areas at 30 days of implantation time ($p < 0.05$) (**Table 1**). In the defect region, the medullar callus (callus behind the defect) was significantly larger than the cortical callus (callus above the defect) at the shorter implantation times of 30 and 60 days ($p < 0.05$) (**Table 2**). As expected, the medullar callus at 30 days post implantation, was smaller than in the implantation time of 60 days and 120 days ($p < 0.05$). Concerning the cortical callus, it also increased with the implantation time: its mean size, for the implantation time of 30 days, was smaller than its mean size for the implantation times of 120 and 180 days ($p < 0.05$); and the mean size for the implantation time of 60 days was significantly smaller than the one observed for the implantation time of 180 days ($p < 0.05$) (**Table 2**). In all samples at different implantation times, it was demonstrated that the callus formation was observed beyond the defect region, both in the medullar and cortical side. The mean size of endosteum callus on the neighboring zone of the defect, with a 1000 μm width, was larger than the cortical callus, on the neighboring zone of the defect, for the implantation time of 30 days ($p < 0.05$) and 60 days ($p < 0.05$) (**Table 3**). The endosteum callus, on the neighboring zone of the defect, also increased as implantation time progressed: at 30 days it was smaller compared to the ones measured for the implantation times of 120 days and 180 days ($p < 0.05$). The periosteum callus, on the neighboring zone of the defect, showed a similar behavior, increasing with the implantation time. Its mean size at implantation time of 30 days was smaller than the mean size for the implantation time of 120 and 180 days ($p < 0.05$) and the mean size for implantation time of 60 days was significantly smaller than for the implantation time of 180 days ($p < 0.05$) (**Table 3**). The mean area fraction occupied by bone lacunae, for the implantation time of 30 days, on samples with a filling percentage greater than 50%, was greater than in other experimental groups, including the group of samples with a filling percentage lower than 50%, for the implantation time of 30 days (**Table 4**).

3 – Discussion:

The sheep model for bone regeneration evaluation shows several advantages when used in pre-clinical studies, such as cost, availability, ethical acceptability, body weight, easy housing and handling. Although goats have similar advantages, the sheep were preferred for these studies, since goats tend to have a more inquisitive and interactive nature, which could turn confinement for long periods more challenging. This was considered as a very relevant aspect for our research studies, as sheep (group 2) had to be transferred to individual cages after implantation procedures, for the first 3 post-surgical weeks, due to the occurrence of a high incidence of post-surgical fractures in the operated femurs in an initial phase (group 1). In subsequent surgeries, the transfer of sheep to individual cages (group 2) drastically decreased the incidence of fractures (5% of fractures). It is our opinion that the cage floor should have a good straw bed, to increase comfort and ease lying down and getting up. Also, to reduce confinement stress, it is advised that individual cages should be near and within visual contact of other sheep, since sheep are gregarious by nature.

Although sheep cancellous bone models are now well established for the assessment of new bone substitutes, the limited availability of cancellous bone makes it difficult to find multiple comparable sites within the same animal, which may preclude the serial sampling and the test of different biomaterials on the same sheep (18). Some of these models have a limitation on the number of defects that can be performed (17, 34), others need more than one surgical incision per limb (7, 19), with longer operative periods, which may result on a more difficult recovery from anesthesia and in an increment in post-surgical morbidity. To increase the number of defects, some models use more than one bone as implantation site. In these cases, one must take care when planning and designing these studies, considering the differences that exist in bone structure, on bone loading and on the amount of overlying soft tissue, since these factors can influence the healing processes (7, 16-19). Bouré, Zeiter (18) developed a sheep model allowing the use of 10 defects with 5 mm diameter: 3 defects in the distal femur and 2 defects in the proximal tibia in each hind limb. In this surgical technique, the access requires the incision of collateral ligament and the surgery area is near the femuro-tibial joint; the identification of this joint must be done with needles to prevent its damage. Possible complications resulting from this technique are the penetration of implants in intercondylar notch and the risk of development of femuro-tibial septic arthritis.

The model here proposed allows creating 10 to 12 defects per sheep, in the same bone with equal conditions of loading, bone structure and amounts of overlying soft tissue. Therefore, it is possible to perform serial sampling in the same animal with the same environmental conditions. The surgical technique is relatively simple: for example, only the skin and *fascia lata* are incised, followed by a blunt dissection without risk of damaging important structures. Instead of using cancellous bone, the defects were created in cortical bone.

Actually, as the adult skeleton is composed by 80% of cortical bone and 20% of trabecular bone, with cortical bone accounting for 70 to 85% of long bones strength (20, 35), cortical bone is more prone to damage in natural conditions. Therefore, most biomaterials used as bone substitutes will be applied on cortical bones.

Regarding *in vivo* testing of bone graft substitutes, the higher metabolic activity and bone turnover of cancellous bone, when compared to cortical bone, can result in the reduction of trabecular bone mass some months after the implantation of a biomaterial, independently of its *in vivo* performance. Therefore, the quantification of new bone mass in cancellous defects, as a parameter to evaluate the bone healing potential of a biomaterial, may produce variable results. By contrast, cortical bone mass tends to increase continuously in a similar extent to biomaterial resorption, as they are strongly negatively correlated in this compartment (36).

On this animal model and with healing periods longer than 60 days, the visualization of defects could be difficult during post-mortem preparation. In these cases, the use of X-ray is recommended to evaluate the location of the defect. Another possibility is to cut out the femoral head, remove the medulla, observe into the medullar space and search for the prominence of bone growth from the defect.

The experimental model here purposed, uses a non-critical size defect, because it allowed the performance of multiple serial analyses of a biomaterial within the same *in vivo* conditions; this feature is very important on the initial screening of a biomaterial *in vivo* behavior, enabling to choose the most suitable chemical composition and the best format of the biomaterial (37, 38). Furthermore, non-critical size defect ensures fast and reliable healing process, allowing the observation of the various stages of bone healing and biomaterial degradation (36). It was also suggested shorter implantation times (30 and 60 days), to observe the early events of bone healing and bone regeneration. These shorter implantation periods permitted to understand the biomaterial's performance, comparing controls (empty defects) with the created defects filled with a biomaterial, as observed on

Figure 9. It was also suggested the use of longer implantation periods (90, 120 and 180 days), in order to study and understand the later stage of bone regeneration, as well as the biomaterial reabsorption and degradation.

A defect size of 5 mm has already been used by other authors in sheep models as non-critical defects (7, 18). This size, despite being non-critical, was chosen because the healing process takes long enough time to allow a comparison between studied biomaterials and controls (**Fig. 9**). The results obtained with the unfilled defects (control defects) analysis, confirmed this fact. As a matter of fact, when analyzing the data, it was found that in all of the studied implantation times, there were samples with unfilled areas and that, at implantation time of 30 days, 61.5% of the samples did not present bone bridging between the edges of the defect. Larger defects, or even of critical size, were not chosen in this study because it would not allow a serial evaluation of large number of samples. Using critical defects in this study, the number of animals had to be increased in order to achieve a sufficient universe to test with statistical validity, the studied materials. The increased number of experimental animals, goes against the 3Rs policy, which implies designing the experience towards maximum information with minimum animal use (21). Furthermore, it should not be ignored that when testing a biomaterial, in distinct animals, there is high individual variability which compromises the conclusions obtained. When delineating animal experimentation and choosing an animal model, and seeking to infer on the behavior of different materials, one should implant on each individual under study, negative controls (empty defects) and all the different studied materials, in order to control the individual variability. Thereby, it was considered in the present study multiple non-critical size defects in the same experimental animal. This first approach in testing the biomaterial, allows defining the ideal composition of the material under study and evaluating its biocompatibility. Once the ideal composition is chosen and biocompatibility is analyzed, the material may be used in clinical trials, or if found necessary, previous to clinical trials resort to critical defects, to confirm the results obtained in the first approach with non-critical defects.

To understand the natural bone healing on this type of defect, it was decided to study the defects without biomaterial (control defects), to provide knowledge of the events involved in bone regeneration without the presence of biomaterial. For that propose it was only considered for histological analysis the control defects (without biomaterial) from a large number of experimental animals previously used to test Bonelike® influence on bone regeneration. For 60 days of healing period or higher, and since we are using non-critical

size defects, all samples had communication between edges, and the mean of unfilled area was only 10.34%. Therefore, the shorter healing periods (30 and 60 days) are probably more adequate to evaluate the performance of the biomaterial. On the other hand, longer healing periods are important to evaluate the biomaterial degradation. With these longer implantation times, the performance of the biomaterial can also be assessed, comparing the quality of the bone and evaluating the percentage of callus formation.

Histological analysis of control defects, at the chosen implantation periods, demonstrated all the steps of bone healing – the reabsorption, the modeling and the remodeling phases (39-43). The reabsorption phase represents the initial phase of bone healing, when osteoclasts remove necrotic debris and clean the edges of the defects after the injury (43). In the present study, it was observed the presence of absorption lacunae on the non-operated bone, on the edges of the defect, in samples with shorter implantation times; this feature decreased as the implantation time increased (**Fig. 8A to Fig. 8D**). This prevailed on samples with a more effective osteo-regeneration, and can be explained by the existence, in these cases, of a higher cellular activity of both osteoblasts and osteoclasts. At implantation time of 30 days, it was verified that, in the defect, osteoblasts secreted collagen fibrils in a 360 degree angle, in random array, to form the woven bone with a randomly oriented matrix deposition, and this new bone had a trabecular pattern, with small amounts of bone matrix and a large number of osteocyte lacunae clusters, close to each other (**Fig. 8A**). This first woven bone serves as a structural scaffold (40, 44). As bone healing progresses the osteoblasts are organized and aligned in a single array along the woven bone surface, and when enough woven bone is secreted, osteoblasts begin to deposit a new matrix in a lamellar orientation, and with this the woven bone is modeled to lamellar bone. This first lamellar bone is formed in a concentric manner, in right angles to the longitudinal axis (**Fig. 8F and Fig. 8G**). This initial perpendicular lamellar orientation appears because, during the healing process, the initial vascular ingrowth is coming from the marrow cavity, and the new lamellar orientation follows this arrangement, perpendicular to the original cortex (40). As the implantation time increased, with the progress of new bone producing, the osteocyte lacunae were surrounded by more bone and the distance between them increased (**Fig. 8G and Fig. 8H**). At implantation time of 180 days, the number and distance between the osteocyte lacunae approached the normal pattern (**Fig. 8H**). At this implantation time it was observed the beginning of the remodeling process, where the lamellar bone formed in a

concentric manner, in right angles to the longitudinal axis, remodeled to a lamellar conformation parallel to the longitudinal axis (longitudinal oriented osteons) (**Fig. 8H**). In the described study it is suggested that, on the defect region, the medullar callus develops faster than the outside callus. With the medullar callus, statistically differences were found between implantation times of 30 and 60 days, and concerning the outside callus the difference is evident between implantation times of 30 and 120 days. For the implantation times of 30 and 60 days, the medullar callus was larger than outside callus, but for the implantation time of 180 days there were no differences in the size between the medullar callus and outside callus. These observations are consistent with Shapiro (39, 40) who describes that in the healing process, the bone formation begins associated to the ingrowth of a rich vascular network from the marrow cavity, and in the first stages of this process there is no bone formation from the periosteum. Also, it is referred that in the repair of a stable and fixed discontinuity with a gap greater than 0.1 mm, the bone marrow stem cells are the primary repair source moving into the interfragmentary gap. For the implantation time of 180 days, the size of medullar callus begins to decrease and this is probably due to the beginning of the remodeling process to restore the original shape of the bone; this last stage of healing is longer and occurs later than the previous ones (45). In the sheep model, in order to clearly see this stage of the healing process, implantation times greater than 180 days are needed. This experimental work suggests that this last stage of bone regeneration begins on the medullar side, probably due to a higher remodeling activity on the endosteal surface than on the periosteal surface, but also to a greater cytokine exposure from the adjacent bone marrow compartment (20) and because almost all osteoclasts arise from the bone marrow monocyte-macrophage precursor cells (20, 45). Our data shows that callus formation extends besides the defect region, both in the medullar and in the cortical sides. The endosteum and periosteum calluses increase the stability of the repair site. During this work, the endosteum callus on the defect neighboring zone, like on the defect zone, were bigger than the periosteum callus, for the implantation times of 30 days ($p<0.05$), 60 days ($p<0.05$) and 120 days ($p<0.1$). The endosteum callus on the defect neighboring zone, unlike the defect zone, did not begin to decrease with the implantation time of 180 days, and in this group, it was still higher than with the implantation time of 30 days. Probably, for bone to acquire the original format in this zone, the remodeling process starts later than in the defect region. When evaluating the bone lacuna percentage of the new bone on the defect, attention should be drawn to separate samples with less than 50% of unfilled area since these

samples have less lacunas than samples with more than 50% of unfilled area ($p < 0.05$) (15.27% and 40.70% respectively for the implantation time of 30 days, (**Table 5**). This probably occurs, because samples with a large unfilled area have small amounts of new bone and this new bone is near the edges of the defect adjacent to membrane layers of mesenchymal cells, which differentiate into osteoblasts, leading to a larger number of osteoblasts and consequently, more bone production and less lacunas spaces. With 180 days implantation time, the percentage of lacunas in our data (3.54%) reaches values that were described for normal compact bone (less than 5%) (20).

With the *Threshold* method analysis, it was proved that it is possible to select and isolate the tissue fraction in study (unfilled zone, lacunae, bone and biomaterial), to calculate areas, allowing to follow the evolution of bone regeneration during a defined healing period. The parameters collected give information on biomaterial's performance, comparing data obtained in controls with biomaterial samples (**Fig. 9**). The rate of biomaterial degradation can also be achieved using the *Threshold* method, by comparing the variation of area occupied by biomaterial, along implantation periods. The *Threshold* method showed to be a reliable and objective histomorphometric method to evaluate biomaterial's performance.

Complementary to the histomorphometric analysis, a histological analysis was also performed which allowed assessing bone quality and interaction between biomaterial and bone. With non-critical size defects purposed in this model, it was possible to observe all bone healing phases, the initial formation of woven bone and trabecular bone (**Fig. 10A**), followed by modeling and remodeling process (**Fig. 10B to Fig. 10E**).

Based on the histological analysis, the biomaterial can be classified as: toxic, when a foreign body reaction with an extensive inflammation is present; bio-inert, when a thin non-adherent fibrous layer forms at the interface between the biomaterial and bone, without inflammatory reactions; bioactive, when a strong and direct link between the bone and the biomaterial is formed, without a mediation of fibrous tissue layer (**Fig. 10B, Fig. 10C, Fig. 10D and Fig. 10E**); and as bioresorbable, when the biomaterial is dissolved / resorbed and gradually replaced by the new bone (**Fig. 10C and 10 E**) (46-48). Considering this analysis, the type of biomaterial degradation can be observed and characterized by the visualization of the biomaterial fragmentation (**Fig. 10D**), the bone invasion into the particles of the material (**Fig. 10C and Fig. 10E**) and the presence of osteoclast on absorption lacunae near de biomaterial (**Fig. 10F**). The evaluation of bone quality also allowed to evaluate the biomaterial performance, comparing different

compositions of the studied biomaterials, between them and the control, as demonstrated in **Fig. 10**, where the control sample presented bone with a trabecular bone pattern (**Fig. 10A**), and the sample with biomaterial already adapted to a lamellar bone pattern (**Fig. 10B**). These histological analyses also allowed visualizing the type of cells present, such as inflammatory cells, osteoblasts, osteocytes and osteoclasts (**Fig. 10F**).

4 – Conclusions:

Although no species fulfils the requirements of an ideal animal model, sheep have several advantages in bone regeneration research. This model allows serial sampling in the same animal with similar environmental conditions. The non-critical size defect chosen for this experimental model permitted evaluating all the events of bone healing, in shorter implantation periods. The surgical technique used is simple, and usually well tolerated by the experimental animals. Based on the data from a large number of control defects (empty holes) from several experimental animals used to test the bone substitute Bonelike® for bone regeneration, it can be concluded that the computer image segmentation test (*Threshold* method) here described is an accurate, simple and fast method to perform the histomorphometric analysis. The *Threshold* method allowed separating and measuring fraction areas of the lacunae, new bone, biomaterial and unfilled zone. The evolution of bone healing could also be evaluated with histological analysis using the purposed animal model. It can also be concluded that the studies with shorter implantation times (less than 60 days) are more adequate for studying the performance of most biomaterials. On the other hand, longer implantations times may be required to evaluate biomaterial degradation.

Legends to the figures, tables and movies

Figure 1: Surgical procedure, the red dashed line represents the skin incision site (cranial edge of the femur) (A); skin incision (B). To visualize the femur diaphysis, the *fascia lata* is incised and *vastus laterallis* muscle is cranially retracted. The *biceps femoral* muscle is caudally retracted with a blunt dissection (C and D).

Figure 2: The performed holes in the femur diaphysis (blue arrow), it can be observed the *fascia lata* over the *biceps femoral* muscle (yellow arrow) (A). Filling the hole with the biomaterial tested - Bonelike® (B and C). Sheep in an individual cage (D).

Figure 3: The complete histological sample (A); the new bone formatted during the healing period (B); the 5 fractions cropped (C). 1 – defect area, 2 – medullar callus, 3 – cortical callus; 4 – the endosteum callus on the defect neighboring zone, with a 1000 μm width, and 5 – the periosteum callus on the defect neighboring zone, with a 1000 μm width.

Figure 4: Using the information obtained with a histogram, the different areas of the studied fractions are separated and measured, including: reabsorption cavity (A), bone (B) and biomaterial (C).

Figure 5: Example of the method to calculate the cutting levels using a histogram: in this example, the medium peak of the histogram is 128 (A); calculation of the cutting level for the reabsorption cavity (black pixels), halfway between 0 and the medium peak of the histogram, $128:2=64$ (B); calculation of the cutting level for the bone (grey pixels), halfway the medium peak of the histogram and the maximum threshold value, $(255-128):2+128=192.5$ (C); cutting level for the biomaterial (white pixels) (D).

Figure 6: *Post mortem* X-rays obtained after a healing period of 60 days (A and B), 120 days (C and D) and 180 days (E and F). For each healing period it was performed a lateral projection (A, C and E) and a dorso-plantar projection (B, D and F). White arrow indicates the location of the empty hole (negative control). The empty hole on C and D is difficult to distinguish and it cannot be differentiated in panels E and F concerning a healing period of 180 days.

Figure 7: *Post-mortem* femur preparation and respective X-ray in a lateral projection. Implantation time of 30 days (A); implantation time of 180 days (B).

Figure 8: Bone healing – reabsorption, modeling and remodeling. Histological images of all study implantation times, stained with Solochrome Cyanine R. NB – new bone, UB – non-operated bone, TB – trabecular bone, LBR – lamellar bone pattern at the right angles to the longitudinal axis of the femur, LBL – lamellar bone pattern parallel to the longitudinal axis, yellow arrow – cluster osteocytes. Absorption lacunae at non-operated bone (A and B). The absorption lacunae on un-operated bone disappeared with the

increase of implantation time (C and D). Bone with trabecular pattern (E). Bone molding to lamellar pattern at the right angles to the longitudinal axis of the femur (F and G). Bone remodeling to a lamellar pattern parallel to the longitudinal axis (H).

Figure 9: *Threshold* method to evaluate the performance of biomaterial. SEM images obtained after a 30 days healing period of an empty defect (control) (**A and C**) and a defect implanted with Bonelike pellets of 250-500µm (**B and D**), on the same animal. On **C and D**, the new bone was selected with the *Threshold* method. It can be noticed more new bone on the sample with biomaterial (**B and D**).

Figure 10: Histological analyses to evaluate the performance and biomaterial interaction. Samples stained with Solochrome Cyanine R (**A, B, C, D and E**) and Hematoxylin & Eosin (**F**), at implantation times of 30 days (**A and B**) and 180 days (**C, D, E and F**), control (unfilled defect) (**A**) Bonelike[®] pellets 500-100µm (BL-sph) (**B, C and E**), Bonelike[®] polygonal (BL-polyg) (**D**). **A** – Control with a trabecular bone pattern (TB). **B** – The bone molded to lamellar bone conformation with a right angle to the longitudinal axis (LBR). **C** – Bone remodeling to a lamellar pattern parallel to the longitudinal axis (LBL) and bone invasion into the biomaterial. **D** – Fragmentation of the biomaterial. **E** – Bone invasion into the biomaterial. **F** – Osteoclasts (red arrow), osteoblasts (yellow arrow) and osteocytes (green arrow).

Table 1: Percentage of unfilled area; means that were significantly different are labeled with different letters (p<0.05).

Table 2: Size of medullar callus and cortex callus in relation to size of the defect (callus area / defect area x 100). On rows the means with * were significantly different for a p<0.05, and with + approaches to significant level p<0.1. On columns, means that were significantly different are labeled with different letters p<0.05.

Table 3: Size of the callus on the neighboring zone of the defect with a 1000 µm width (callus area / defect area x 100). On rows the means with * were significantly different for a p<0.05, and with + approaches to significant level p<0.1. On columns, means that were significantly different are labeled with different letters p<0.05.

Table 4: Percentage of bone lacunae; * samples with a percentage of defect filling <50%; ** samples with a percentage of defect filling >50%. Means that were significantly different are labeled with different letters (p<0.05).

Movie 1 – Convert image to 8 bit scale.

Movie 2 – Threshold analysis with image J software.

Movie 3 – Selection of the lacunae with the aid of histogram.

Movie 4 - Selection of the new bone with the aid of histogram.

Movie 5 - Selection of the biomaterial with the aid of histogram

6. Acknowledgments

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7. Disclosure statement

Authors declare that the manuscript does not have any commercial association that might create a conflict of interest. Also, any competing financial interests, actual or potential, of each author has been appropriately disclosed.

Figures:

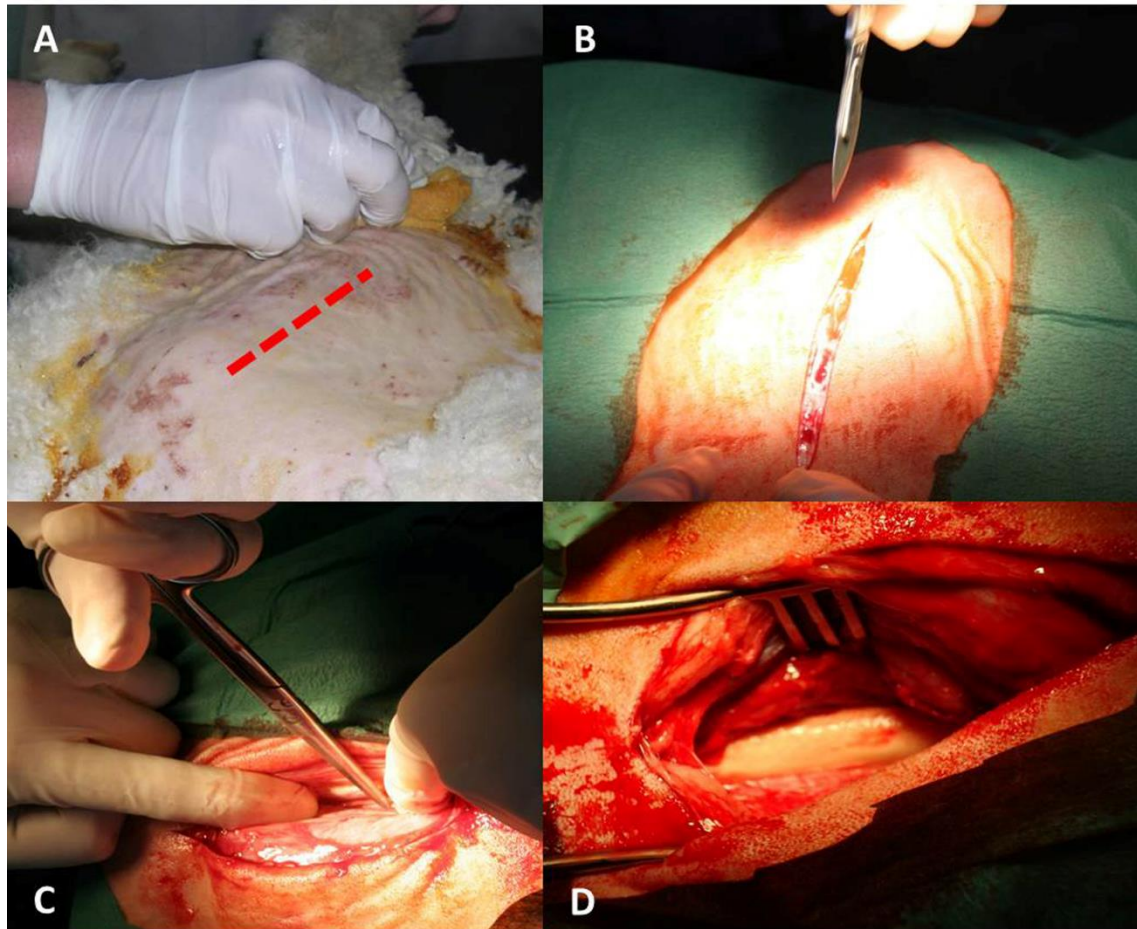


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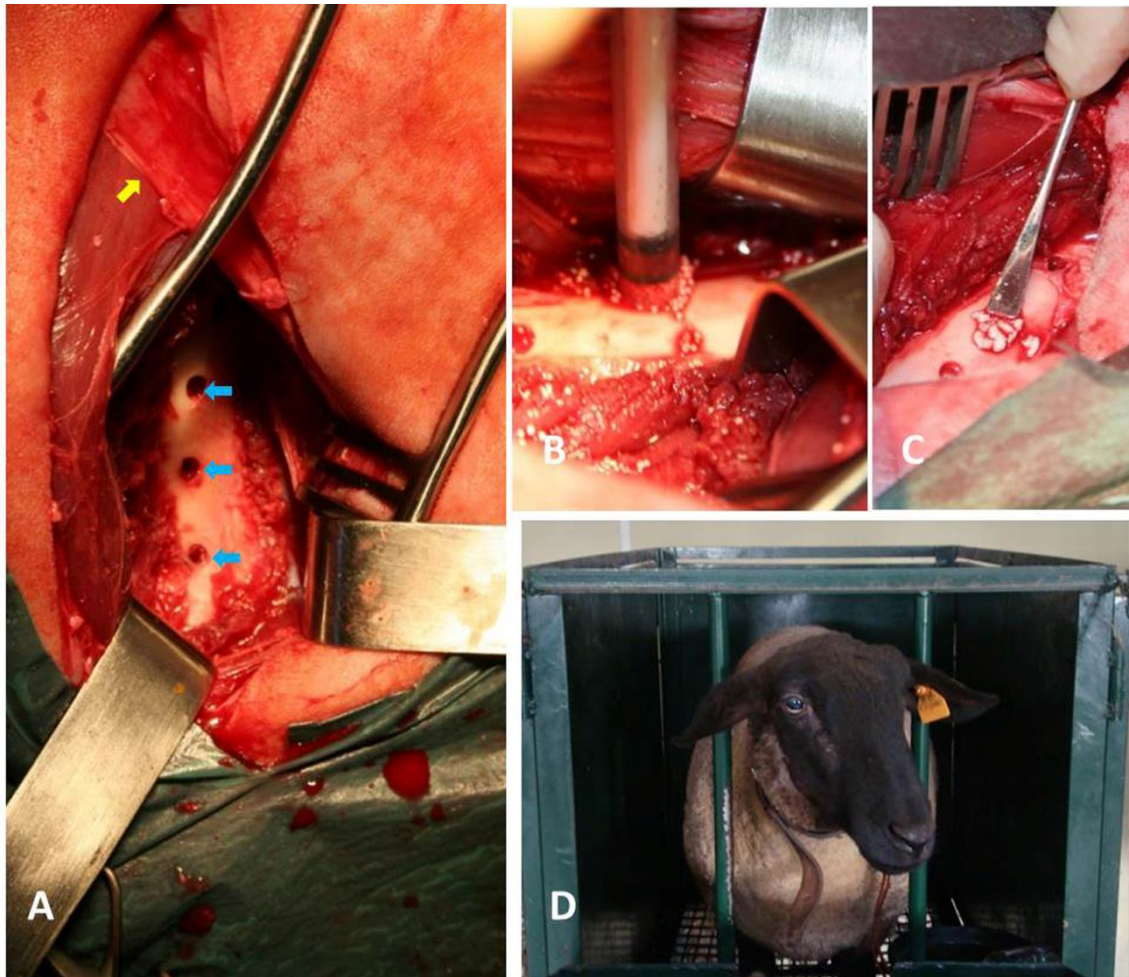


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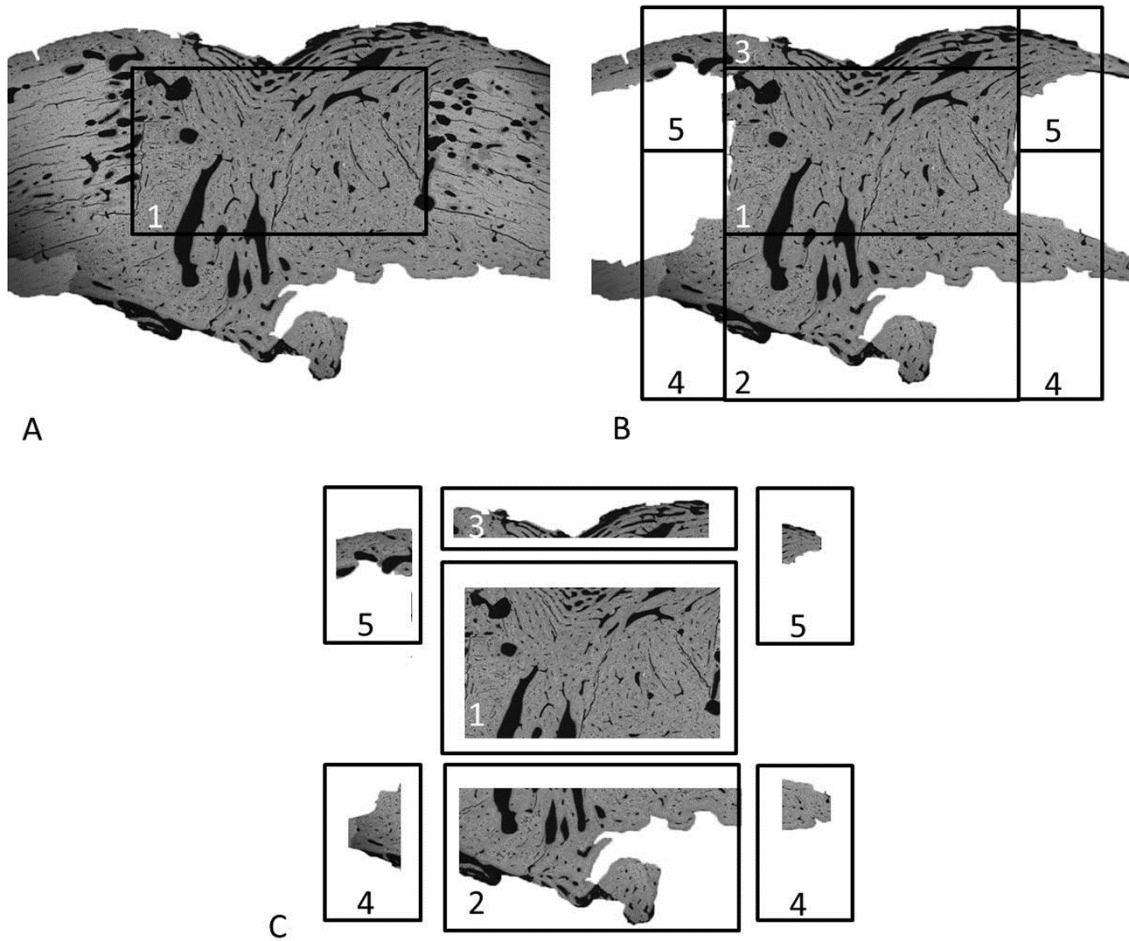


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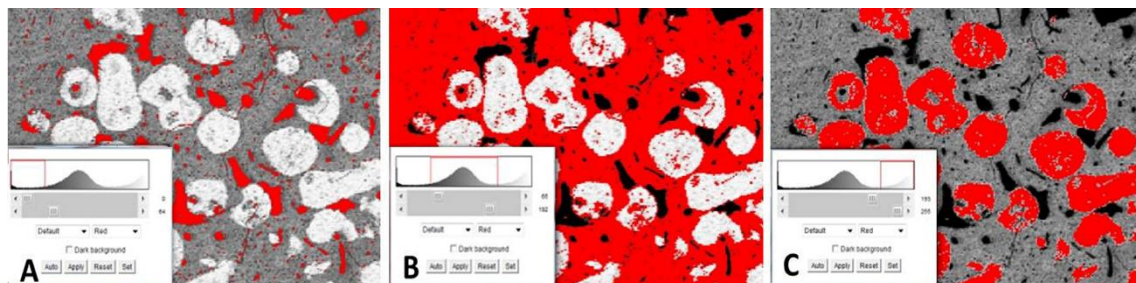


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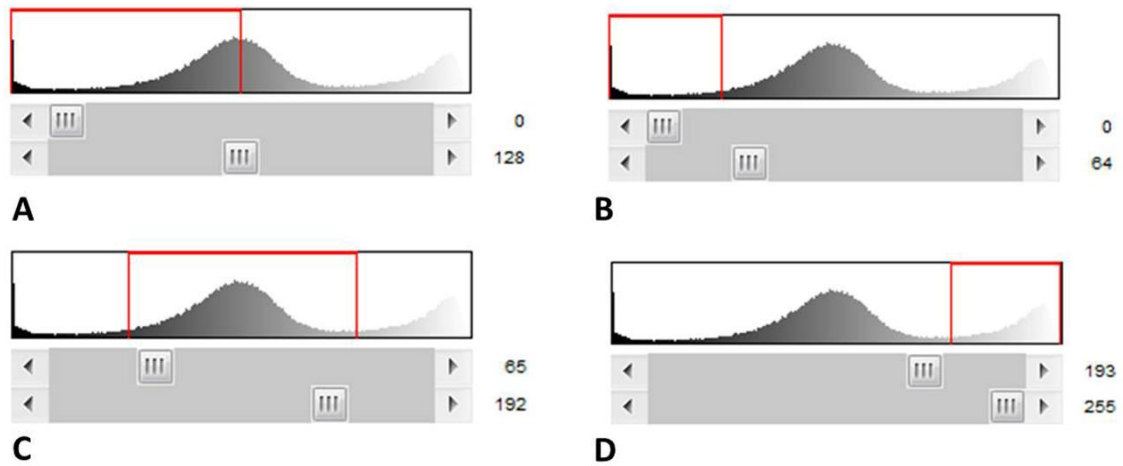


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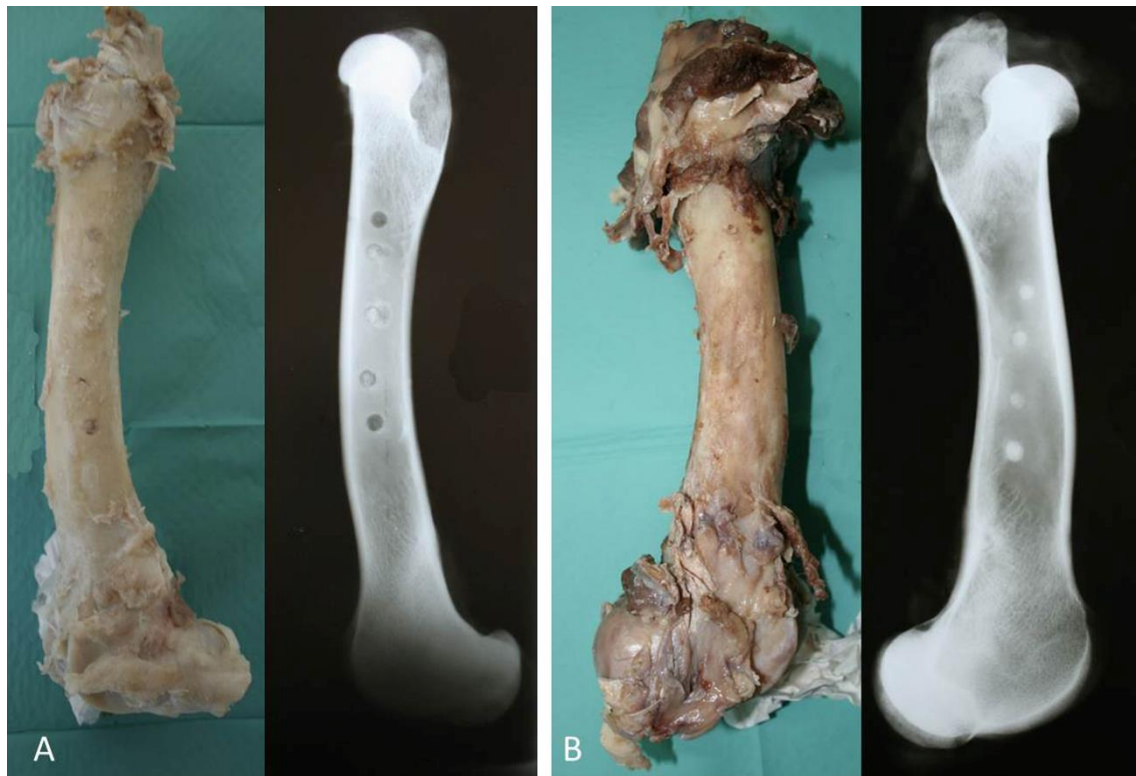


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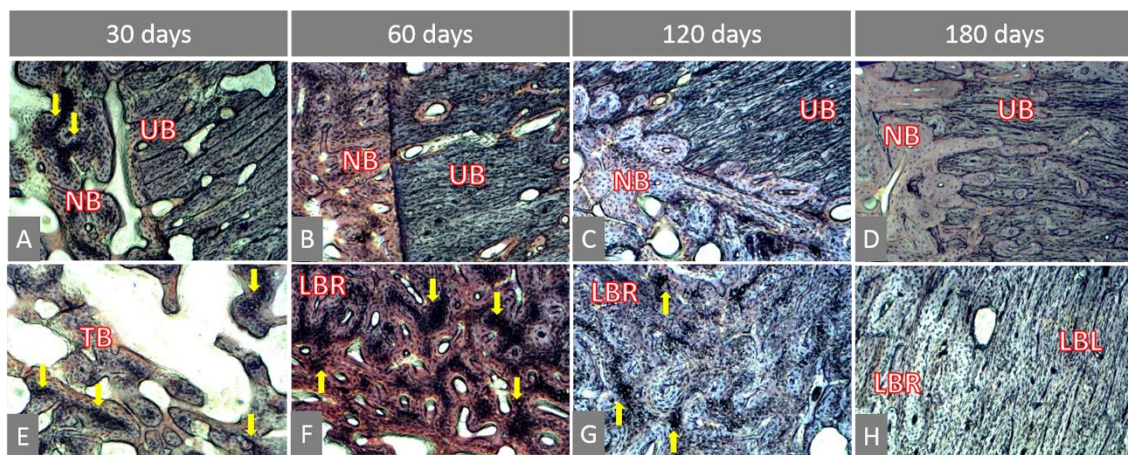


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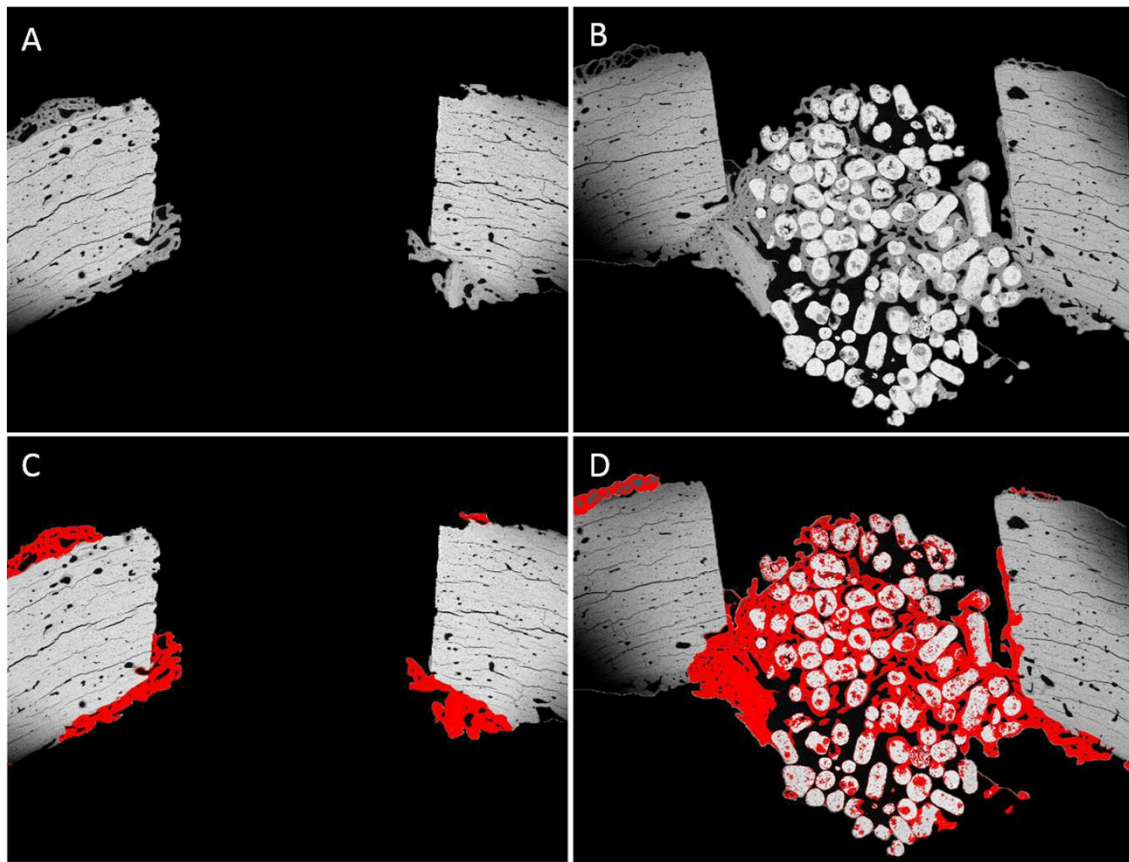


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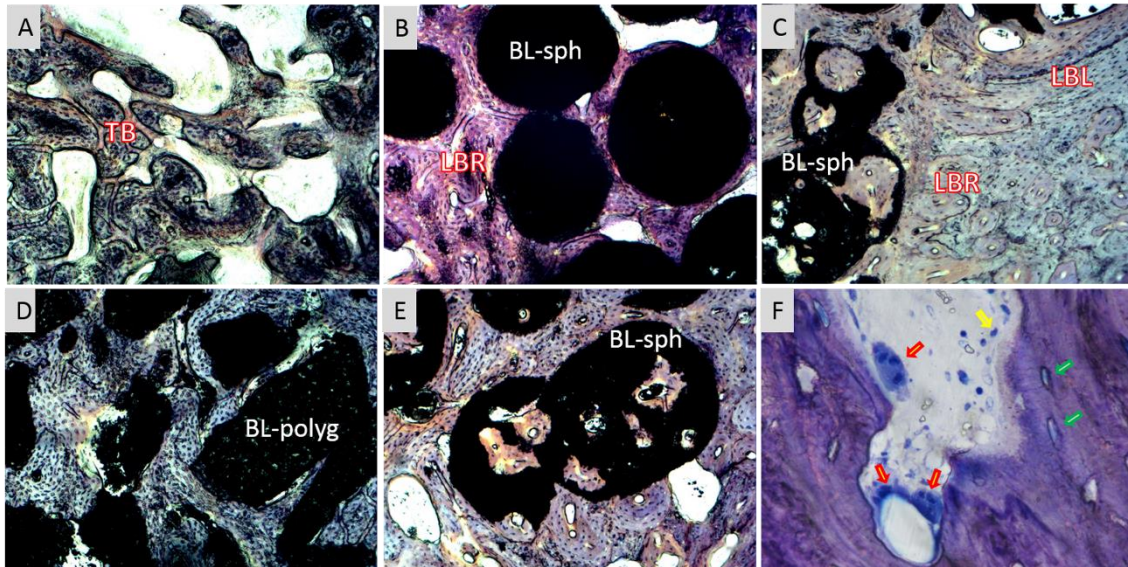


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Table 1:

Implantation time	mean unfilled area	sd	min	max
30 days (N=13)	52.91% ^a	40.41	0	99.58
60 days (N=4)	10.34% ^{ab}	12.19	0	23.68
120 days (N=7)	1.74% ^b	2.54	0	7.3
180 days (N=5)	0.87% ^b	1.94	0	4.35

Table 1: Percentage of unfilled area; means that were significantly different are labeled with different letters (p<0.05).

Table 2:

defect region	callus area / defect area x 100							
	Medullar (inside) callus				Cortical (outside) callus			
Implantation time	mean	s d	min	max	mean	s d	min	max
30 days	14.43^{*a}	17.39	0.00	55.12	1.93^{*c}	3.39	0.00	10.26
60 days	35.43^{*b}	13.72	16.2	47.45	8.34^{*cd}	7.25	2.02	16.15
120 days	36.94^{+b}	23.74	10.56	64.9	17.43^{+de}	7.30	5.95	27.63
180 days	23.84^{ab}	8.69	12.4	34.49	25.29^e	17.99	0.00	48.23

Table 2: Size of medullar callus and cortex callus in relation to size of the defect (callus area / defect area x 100). On rows the means with * were significantly different for a $p < 0.05$, and with + approaches to significant level $p < 0.1$. On columns, means that were significantly different are labeled with different letters $p < 0.05$.

Table 3:

neighboring zone defect (1000 μ m width)	callus area / defect area x 100							
	endosteum callus				periosteum callus			
Implantation time	mean	s d	min	max	mean	s d	min	max
30 days	12.88^{*a}	8.46	7.77	17.99	3.59^{*a}	4.59	0.00	16.38
60 days	17.38^{*ab}	5.57	8.51	26.25	8.92^{*ab}	3.29	5.29	13.02
120 days	18.84^{+bc}	6.18	13.13	24.55	12.66^{+bc}	5.84	4.4	23.37
180 days	19.14^c	4.93	13.02	25.26	18.9^c	8.5	8.94	29.39

Table 3: Size of the callus on the neighboring zone of the defect with a 1000 μ m width (callus area / defect area x 100). On rows the means with * were significantly different for a $p < 0.05$, and with + approaches to significant level $p < 0.1$. On columns, means that were significantly different are labeled with different letters $p < 0.05$.

Table 4:

bone lacunae percentage of the defect				
Implantation time	mean	s d	min	max
30 days*	15.27% ^a	5.16	9.61	20.92
30 days**	40.70% ^b	13.20	25.02	63.30
60 days	14.94% ^b	1.57	13.85	17.21
120 days	12.97% ^b	7.25	6.82	25.61
180 days	3.54% ^b	3.41	0.20	8.33

Table 4: Percentage of bone lacunae; * samples with a percentage of defect filling <50%; ** samples with a percentage of defect filling >50%. Means that were significantly different are labeled with different letters (p<0.05).

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Capítulo 4

Bonelike[®] esférico (*pellets*)

4 – Bonelike[®] esférico (*pellets*)

Neste capítulo apresentam-se os resultados da caracterização da nova formulação do Bonelike[®] esférico com duas granulometrias, nomeadamente 250-500µm e 500-1000µm. Nestes trabalhos, além de se avaliar o comportamento *in vivo* desta nova formulação, estudou-se a influência da forma física, estrutura e microestrutura das diferentes formulações e granulometrias de Bonelike[®] na regeneração óssea.

Publicações

Artigos em Revistas Científicas e Indexadas

P.P. Cortez*, L.M. Atayde*, M.A. Silva, P.A.S. Armada-da-Silva, M.H. Fernandes, A. Afonso, M.A. Lopes, A.C. Maurício and J.D. Santos (2011). "Characterization and preliminary in vivo evaluation of a novel modified hydroxyapatite produced by extrusion and spheronization techniques." Journal of Biomedical Materials Research Part B: Applied Biomaterials 99B(1): 170-179. **These authors contributed equally to this work.*

L.M. Atayde, P.P. Cortez, A. Afonso, M. Santos, A.C. Maurício and J.D. Santos (2014). "Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike[®]) on osteoregeneration" Journal of Biomedical Materials Research Part B: Applied Biomaterials (*submetido*).

Comunicações Orais em Congressos

J. D. Santos, L. M. Atayde*: "Biomaterials and 3D Biomodelling for Bone Regenerative Medicine" (2010) 1st International Meeting of Microsurgical Societies, Genoa – Italy May 20-22. ** Presenting author*

**Characterization and preliminary in vivo evaluation of a novel
modified hydroxyapatite produced by extrusion and
spheronization techniques.**

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Characterization and preliminary *in vivo* evaluation of a novel modified hydroxyapatite produced by extrusion and spheronization techniques

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Abstract: A glass-reinforced hydroxyapatite (HA) composite, recently registered as Bonelike[®], was developed for bone grafting. This biomaterial is composed of a modified HA matrix with α - and β -tricalcium phosphate secondary phases and ionic species that mimic the chemical composition of human bone. Several *in vitro* and *in vivo* studies have confirmed the benefits of these properties. However, these studies were all executed with Bonelike[®] polygonal granules obtained by crushing. In this study, Bonelike[®] pellets were produced through a patented process, which required the use of techniques such as extrusion and spheronization. The final product presented a homogeneous size, a 55.1% global porosity and a spherical shape. This spherical shape permitted a better adaptation to the implantation site and improved injectability. Additionally,

it also may contribute to formation of macropores as pellets packaging leaves open spaces. After implantation of Bonelike[®] polygonal granules and Bonelike[®] pellets in monocortical defects in sheep for 8 and 12 weeks, light microscopy and scanning electron microscopy showed extensive osteointegration simultaneously with bone regeneration for both presentations. Histomorphometric analysis did not reveal statistically significant differences between defects treated with Bonelike[®] polygonal granules and Bonelike[®] pellets, which suggests similar *in vivo* performances. © 2011 Wiley Periodicals, Inc. *J Biomed Mater Res Part B: Appl Biomater* 99B: 170–179, 2011.

Key Words: bioactive glass, *in vivo*, hydroxyapatite, biocompatibility/hard tissue, bone graft

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INTRODUCTION

Bone grafts are the second most transplanted material, surpassed only by blood.¹ The consensual “gold standard” graft remains the autologous graft, which does not induce immunological reactions and has the ability to provide osteoinductive growth factors, osteogenic cells, and structural scaffolds.² However, it requires an additional incision site, leading to donor site morbidity, to a secondary functional deficit, and to blood loss.^{3,4} Concerning allografts, there is the risk of transferring viral infections, such as HIV, hepatitis B, hepatitis C, and prionic infections implicated in a number of diseases like the Creutzfeldt-Jakob disease in humans.

Also, the clinical use of allografts increases the risk of immunological reactions.^{3,4} Thus, several synthetic bone grafts have been developed and used clinically to provide a more secure alternative to autografts and allografts.⁵ A wide variety of commercial synthetic forms of bone grafts based on hydroxyapatite (HA) is available on the market, because of its chemical similarity to the mineral phase of bone.² HA has the ability to bond and integrate with the host tissue when implanted, but its slow resorption rate may restrict bone formation directly on its surface and, subsequently, compromise a proper remodeling of the newly formed bone, resulting in a significant reduction of its strength.⁶

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Over the past decade, the authors developed a glass-reinforced HA composite, recently registered as Bonelike[®].^{7–11} Bonelike[®] is composed of modified calcium phosphates with controlled percentages of ionic species, such as magnesium, sodium, and fluoride^{12,13} that mimic the chemical composition of the mineral phase of human bone. The presence of α - and β -tricalcium phosphate (TCP) secondary phases in its composition affords a higher solubility than HA¹⁴ and contributes to the release of the referred ionic species. Additionally, the presence of a vitreous liquid phase during the sintering process allows the formation and homogeneous dispersion of α - and β -TCP phases in the HA matrix. As these phases are biodegraded more rapidly than the matrix, this dispersion assures a homogeneous degradation of Bonelike[®] and, therefore, prevents a too rapid release of microparticles from the material, which could become targets for macrophage phagocytosis.¹⁵

The above-referred properties might explain the significant osteoblast differentiation and proliferation, as well as bone mineralization, observed in *in vitro* studies.^{16–23} Data obtained from *in vivo* animal studies, where bone defects were surgically created, revealed a very satisfactory osteointegration and a sustained controlled resorption of Bonelike[®].^{12,24–29} All these experiments were performed with Bonelike[®] polygonal granules obtained by crushing, which consist of irregular fragments with a great variability in the size and the degree of porosity, because of the destruction of the surface layer during the crushing procedures.³⁰ Additionally, these granules have sharp areas that may adversely affect osteogenesis.³¹

This study was performed using Bonelike[®] pellets obtained through a patented process,³² which requires the use of techniques such as extrusion and spheronization. Bonelike[®] pellets present a spherical shape and a regular size that facilitates the manipulation by the surgeon and improves their injectability for local applications,^{33,34} because of their more predictable flowing properties.^{35–38} Furthermore, this shape permits a better adaptation to irregular implantation sites³⁸ and may prevent agglomeration of granules after application, contributing to the formation of macropores as pellets packaging results in open spaces. The subsequent bone ingrowth can also be enhanced by micropores in the surface of the pellets, originated from the production method, which facilitate cell adhesion and promote newly formed bone apposition.

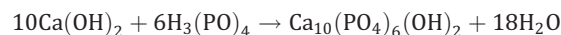
The aim of this experimental work was to assess the physicochemical properties of Bonelike[®] pellets and to compare the bone healing potential in femoral diaphyseal defects, between Bonelike[®] pellets and Bonelike[®] polygonal granules, in the sheep model.

MATERIALS AND METHODS

Material preparation

The preparation of phase pure HA was performed using a chemical route. Hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] preparation required the precipitation between calcium hydroxide [$\text{Ca}(\text{OH})_2$] (Prolabo, Paris, France) and orthophosphoric acid

85 wt%, [H_3PO_4] (Merck, Darmstadt, Germany), according to the following chemical reaction:



Filtered and dried HA precipitate was ground into a fine powder, with a granulometry less than 75 μm . A P_2O_5 -CaO-based glass with the composition of 65 P_2O_5 -15CaO-10 CaF_2 -10 Na_2O (mol %) was prepared by mixing reagent-grade chemicals: disodium carbonate (Na_2CO_3 ; Panreac, Barcelona, Spain), calcium fluoride (CaF_2 ; Merck, Darmstadt, Germany), calcium hydrogen phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$; Sigma, St. Louis, USA), and phosphorus pentoxide (P_2O_5 ; Panreac). A frit was obtained after heating the mixture at 1450°C for 30 min in a platinum crucible. The prepared glass was crushed in an agate mortar and sieved to a granule size below 50 μm .

Bonelike[®] was obtained by adding 2.5 wt % of glass to pure phase of prepared HA. For the pellets production, Bonelike[®] powder was first mixed with microcrystalline cellulose in a double cone mixer. The mixture was placed on a planetary mixer and purified water was added to the mixture for the paste malaxation. After 5 min, the moist paste was placed on a polyethylene air-deprived double bag for 1 day, resulting in the hydration of the microcrystalline cellulose. Then, the moist paste was extruded in an extruder equipped with an extrusion screen of 1 mm (Caleva Extruder 20, Caleva Process Solutions, Blandford, UK). Following the extrusion process, the extrudate was placed on a spheronizer (Caleva Spheronizer 120, Caleva Process Solutions, Blandford, UK) and after this step, the pellets were removed and dried in a forced air circulation oven at 60°C. These pellets were subsequently heat treated to burn out the organic additive at 600°C, using a ramp rate of 0.5°C/min, and sintered at 1300°C using a ramp rate of 4°C/min. This set temperature was maintained for 1 h, followed by natural cooling inside the furnace. Detailed description of preparation of Bonelike[®] pellets³² and polygonal granules¹¹ has been previously reported. Phase identification and quantification were assessed by X-ray diffraction and Rietveld analysis.

Scanning electron microscopy

Bonelike[®] pellets were examined using a scanning electron microscope (SEM) equipped with an energy-dispersive X-ray spectroscopy system (FEI Quanta 400 FEG ESEM, Hillsboro, USA; EDAX Pegasus X4M, Mahwah, USA) operating in high vacuum mode at an accelerating voltage of 15 kV. On Bonelike[®] pellets surface, this method was used to characterize the macrostructure and microstructure and to qualitatively assess pore morphology.

Density and porosity

Bulk and apparent densities and porosity were determined by mercury porosimetry (AUTO PORE IV 9500, Micromeritics, Aachen, Germany), which is based on the intrusion of mercury under pressure into the sample pores. Diameter of sample pores lower than 360 μm required a pressure

between 0.5 and 25,000 psia (intraporosity). Sample pores larger than 360 μm required a pressure between 25,000 and 60,000 psia (interporosity).

Fourier transformed infrared spectroscopy

Infrared spectra analysis was performed with a Fourier transform infrared (FTIR) system 2000 from Perkin-Elmer, with 4 cm^{-1} resolution and 200 scans. Bonelike[®] powders were mixed with potassium bromide (KBr) and thin discs were prepared under uniaxial pressing.

X-ray diffraction analysis

Rietveld and X-ray diffraction (XRD) analyses were performed to identify and quantify the percentage of crystalline phases present in the microstructure of Bonelike[®] using a Siemens D5000 diffractometer with Cu-K α radiation ($\lambda = 1.5418 \text{ \AA}$). The scans were performed in the range of 24–42°, with a step size of 0.02° and a count time of 2 s/step.

Carbon determination

To determine the presence of organic residues, because of the employment of microcrystalline cellulose on Bonelike[®] pellets manufacture, carbon determination was performed by infrared spectroscopy absorption using an induction furnace (LECO CS-200). Analyses were made using samples with 0.2–0.5 g of material.

In vivo studies

Four healthy skeletally mature Merino breed sheep, with an average weight of 50 kg, were used as experimental models. All procedures were performed with the approval of the veterinary authorities of Portugal in accordance with the European Communities Council Directive 86/609/EEC. Surgeries were conducted under general anesthesia.

In the lateral diaphysis of each sheep femur, a row of five holes with a diameter of 5.0 mm was drilled through the cortex and into the medulla, using a microburr continuously flushed with a sterile normal saline solution to minimize thermal damage and to remove any residual bone. A minimal distance of 1 cm was kept between drill holes to reduce the risk of fracture. Adequate volumes of Bonelike[®] pellets (ranging in size from 500–1000 μm) and Bonelike[®] polygonal granules (500–1000 μm), premixed with an equal part of peripheral blood, were applied to ensure complete filling of the defects. Defects to be used as negative controls were left unfilled. These procedures were repeated on the contralateral leg.

Sheep were then transferred to individual cages and allowed to move without restriction. Three weeks after the surgeries, the animals were transferred to straw yards, remaining there until the end of the implantation periods.

The healing process was followed through X-ray imaging immediately after surgery and repeatedly at 2 weeks intervals until sacrifice.

Three separate fluorochrome labels were administered at 14 days (calcein green, 15 mg/kg), 8 days (alizarin red, 40 mg/kg), and 3 days (oxytetracycline, 40 mg/kg) before sacrifice, by subcutaneous injection, to measure bone appo-

sition rates (i.e., the rate of bone ingrowth or bone deposition) within the granular implants. Fluorochrome labels are bound to sites of active bone deposition shortly after administration.³⁹

An intravenous injection of a 20% pentobarbital sodium solution was used to sacrifice three sheep 8 weeks after the surgeries and one after 12 weeks. Subsequently, femurs were dissected and all soft tissue was stripped from the bones. Segments of these femurs containing the implants were sawed out.

Histological analysis

Samples were immediately placed in a neutral formaldehyde fixative solution (10%) for 7 days, dehydrated in an increased percentage of alcohol solutions, and embedded in a methyl-methacrylate resin. Thin sections with 150 μm were cut perpendicularly to the femur axis with a diamond blade microtome and hand-ground to approximately 70–80 μm . Sections were then stained with Solochrome cyanine R for histological examination with a Nikon light microscope (Eclipse E600, Nikon, Tokyo, Japan), equipped with a calibrated digital camera (Nikon DS-5M-L1 Digital Sight Camera System, Nikon). Unstained slides were studied under an Axiolab A reflected-light fluorescence microscope (Carl Zeiss MicroImaging GmbH, Germany) to detect fluorochrome labels.

Histomorphometric analysis with scanning electron microscopy

Histomorphometric measurements were performed on unstained slides using a SEM (FEI Quanta 400 FEG ESEM) operating in high vacuum mode at an accelerating voltage of 15 kV. SEM was also performed to evaluate formation of new bone within the defects and the osteointegration and degradation of the studied biomaterials.

High-resolution SEM images (35 \times magnification, 4096 \times 3536 pixels) were imported into ImageJ software (v1.41o, National Institute of Health, USA) for histomorphometric analysis. First, the percentage of defect filling in relation to the surrounding nonoperated cortical bone was calculated independently from the other fractions, as it may provide pertinent data concerning the postsurgical fracture risk.⁴⁰ Subsequently, the quantification of area percentage was performed for three different fractions across the new bone: biomaterials, void spaces (e.g., resorption cavities, Haversian canals, and blood vessels), and new bone. As the unfilled defect and void spaces inside the new bone appeared darker, Bonelike[®] particles appeared whiter and new bone consisted of various shades of gray, the corresponding thresholds were set interactively per each image by visual evaluation of the gray level frequency distribution histogram. The area percentage of each fraction was calculated for each image by the computer in a binary image that resulted after the application of a grayscale eight-bit discrimination step, in which the area of interest was derived by the threshold procedure. The pixel count for each fraction was divided by the total number of image pixels and, thus, expressed in percentage of the area occupied by that fraction (Figure 1).

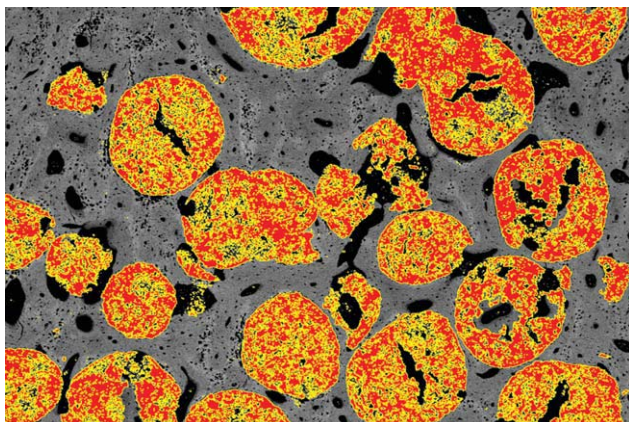


FIGURE 1. SEM image displaying the area occupied by Bonelike[®] pellets (red with yellow outline), selected by the threshold procedure through visual evaluation of the grey level frequency distribution histogram with ImageJ software. The area percentage of each fraction was calculated after dividing the respective pixel count by the total number of image pixels between the defect borders (magnified image for exemplifying purposes). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Statistical analysis

Only data for eight weeks after bone defects implantation were used in the statistical analysis. This data was obtained from histomorphometric analyses of samples collected from three sheep, consisting of nine defects per each treatment: negative controls (defects left unfilled), Bonelike[®] polygonal granules, and Bonelike[®] pellets. Data obtained for area fractions were analyzed through one-way ANOVA using the composition for bone defect repair as the dependent variable. Tukey's HSD test was used for pairwise comparisons. Statistically significant differences were considered for $p < 0.05$.

RESULTS

Physicochemical characterization

XRD analysis of Bonelike[®] pellets revealed that during the sintering process, a reaction between the HA matrix and P_2O_5 -based glass occurred, which originated a final product with a main crystalline phase of HA and with β - and α -TCP as secondary phases. Phase quantification showed that the weight percentage of each phase was: HA = 68%, β -TCP = 19%, and α -TCP = 13%.

SEM analysis revealed that Bonelike[®] pellets presented a homogeneous size distribution, ranging from 500 to 1000 μm , with an average size of 685 μm , and a spherical shape (Figure 2).

The obtained pellets presented a pore distribution depicted in Figure 3, where it was possible to observe intrapores and interpores, the second and first peaks, respectively. The obtained intrapores exhibited interconnected micropores and mesopores (the second peak of Figure 3), with pore diameters lower than 10 μm . Interporosity presented pores with diameters lower than 343 μm .

Extrusion and spheronization processes allowed the production of Bonelike[®] pellets with 55.1% global porosity. Intraporosities and interporosities represented 24.1% and 31.0 % in volume, respectively. These pellets showed a bulk density of 1.34 g/mL and an apparent density of 2.98 g/mL.

FTIR revealed the presence of the groups PO_4^{3-} , OH^- , and H_2O . Hydroxyl groups (OH^-) were detected at 3570 and 630 cm^{-1} . The broad band observed at 3200–3600 cm^{-1} indicated absorbed water on the materials. Phosphate peaks were detected at 566, 597, 958, 1041, and 1086 cm^{-1} .

Infrared spectroscopy absorption analysis demonstrated that organic combustion of cellulose microcrystalline, during the heat treatment at 600°C, was effective, as only very small percentages of carbon were detected: 0.013% on

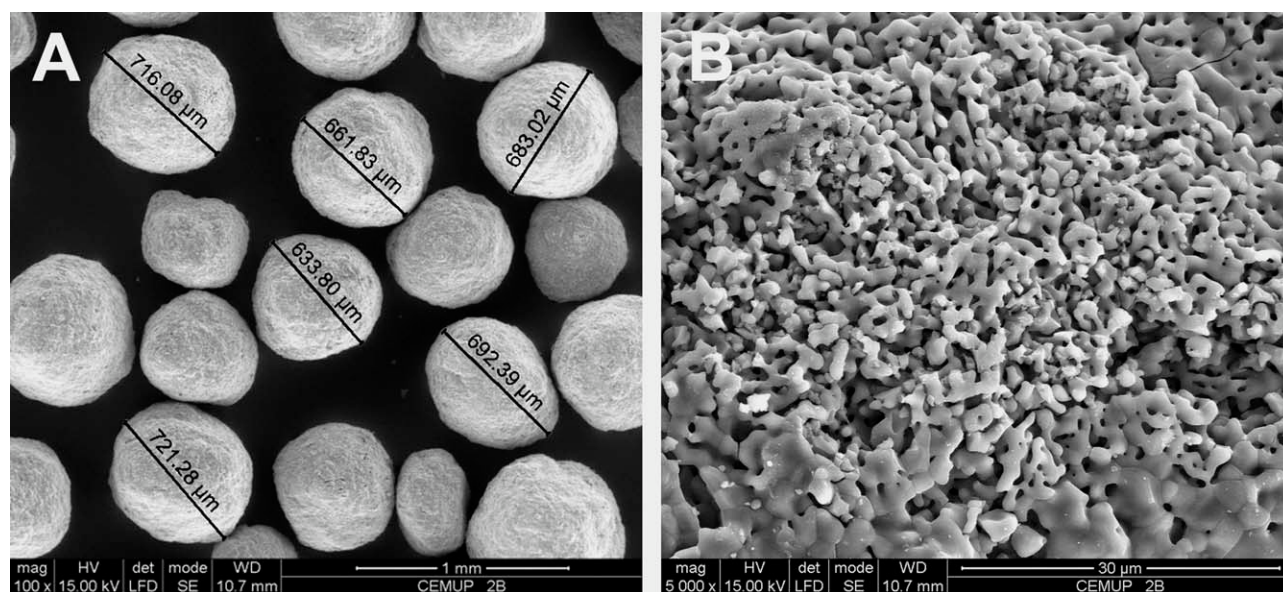


FIGURE 2. SEM images of Bonelike[®] pellets: microscopic appearance (A) and surface detail (B).

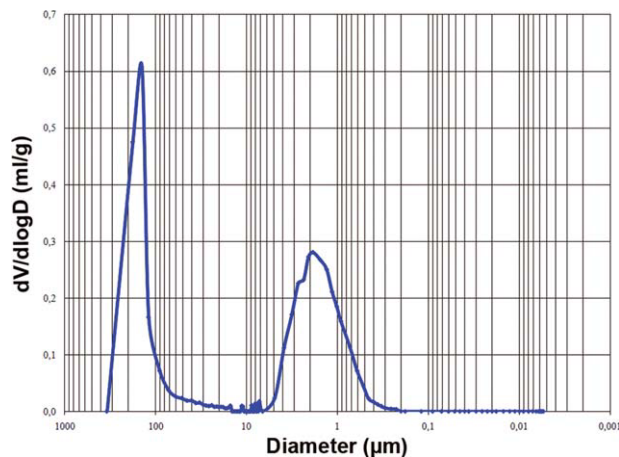


FIGURE 3. Bonelike[®] pellets pore distribution, determined by mercury porosimetry. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

samples sintered on the bottom of the woven and 0.02% of carbon on samples sintered on the top of the woven.

***In vivo* evaluation**

During surgeries, implantation of Bonelike[®] pellets was easier to accomplish than with Bonelike[®] polygonal granules. The former biomaterials were quickly injected with the aid of a syringe.

The four sheep tolerated the bilateral bone grafting procedure and passed the follow-up period without complications. The animals were fully weight-bearing on the first postoperative day.

On the radiographs taken in the first day after surgery, the drill holes were observed as radiopaque circles, if grafted with Bonelike[®] pellets or granules or in radiolucent circles for empty defects. As the implantation period progressed, the radiopacity slightly decreased, which was par-

ticularly evident in the radiographs taken after femurs dissection, at 8 and 12 weeks, suggesting resorption of these ceramics (Figure 4).

New bone formation was confirmed by both histological and SEM analyses for all implant compositions (Figure 5). In this new bone, osteointegration of Bonelike[®] granules and pellets was achieved without local or systemic signs of a foreign body response to the biomaterials. An apparently healthy new bone, with embedded osteocytes adjacent to resorption lacunae, surrounded these biomaterials. Bone remodeling around the granules and pellets was confirmed by the presence of Haversian canals in close proximity to these lacunae. Moreover, signs of biomaterials degradation with simultaneous direct bone apposition, within the granules or directly on their surface, were detected (Figures 6 and 7). In the defects filled with Bonelike[®] polygonal granules, extensive fragmentation and dissolution features were observed inside some resorption lacunae (Figure 8). These degradation features were much less pronounced for Bonelike[®] pellets (Figures 6 and 7). The presence of resorption lacunae appeared more pronounced in samples with 8 weeks, while osteointegration was more marked in samples with 12 weeks. In the latter samples, an apparent decrease in the density of Bonelike[®] particles, along with intensified signs of their degradation, were more noticeable.

Optical fluorescence microscopy demonstrated an intense and direct bone apposition in the bone-to-ceramic direction, defined by the presence of the sequentially administered fluorochrome labels as colored bands inside the Bonelike[®] particles and in the surrounding remodeling lacunae (Figure 9). The presence of Haversian osteonal remodeling around the ceramic materials was also confirmed with this method.

Statistical analysis was performed only in samples with 8 weeks collected from three sheep, consisting of nine defects per each treatment: negative controls (defects left

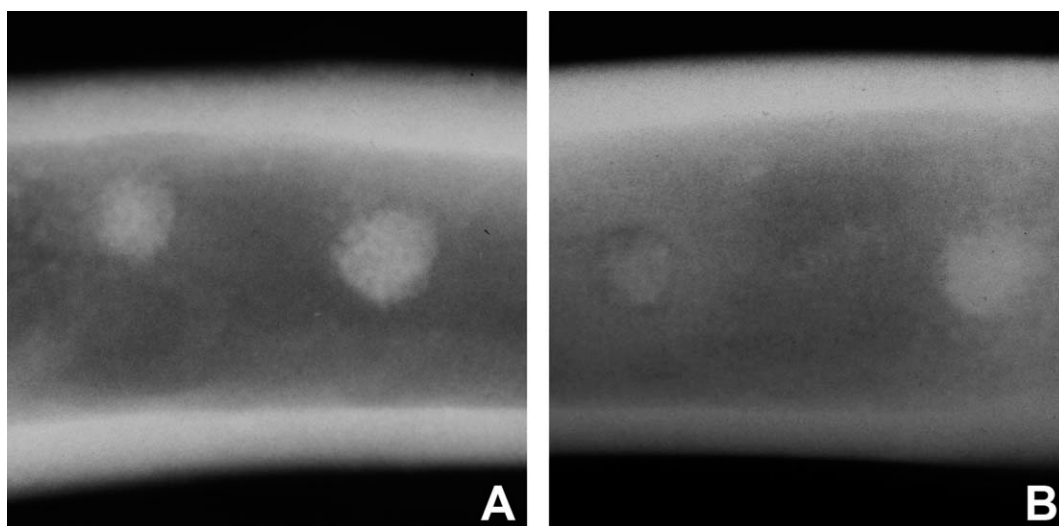


FIGURE 4. Radiographs taken after femurs dissection, showing two defects filled with Bonelike[®] pellets after (A) 8 and (B) 12 weeks of implantation.

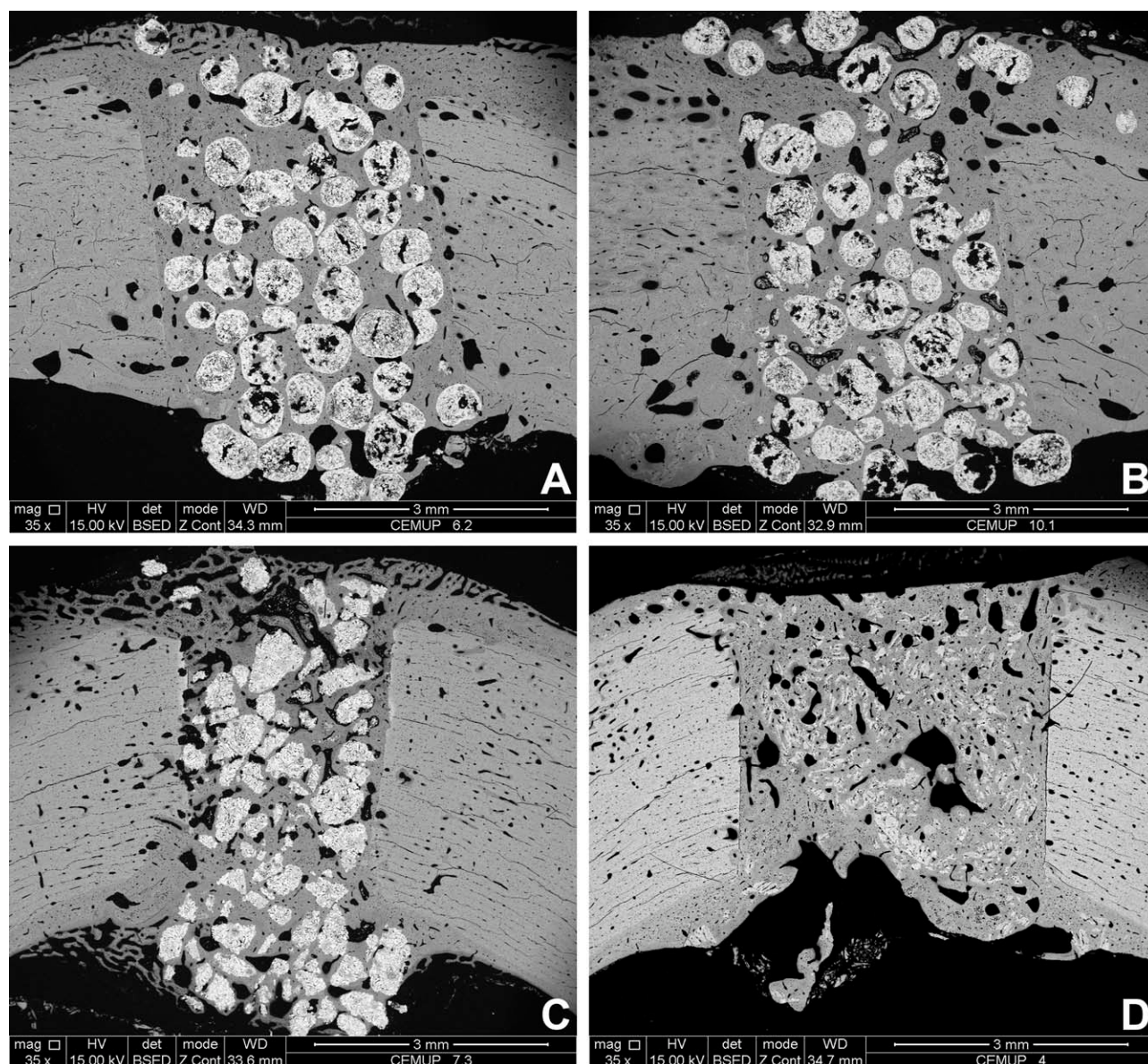


FIGURE 5. SEM images of cortical defects taken at 8 and 12 weeks postimplantation: Bonelike[®] pellets after (A) 8 and (B) 12 weeks of implantation; (C) Bonelike[®] polygonal granules after 8 weeks; (D) negative control after 12 weeks.

unfilled), Bonelike[®] polygonal granules, and Bonelike[®] pellets. The percentage of defect filling was significantly lower in negative controls compared to defects filled with both types of Bonelike[®] ($p < 0.05$). The percentage area of void spaces in negative controls was significantly higher when compared to Bonelike[®] pellets ($p = 0.03$) but not when compared to Bonelike[®] polygonal granules ($p = 0.401$). Negative controls also presented significant higher values of new bone because of the presence of Bonelike[®] particles in the other samples, which limited new bone formation, and due to the fact that noncritical size defects were used in this study, that is, healing would have occurred even without implantation of biomaterials. No significant differences were detected between the two types of Bonelike[®] in any of the measured parameters (Table I).

DISCUSSION

The production process of Bonelike[®] pellets,³² based on the pharmaceutical technology of extrusion and spherization, was chosen because of its low cost, higher reproducibility, higher yield, and productive capacity. The final product presented a homogeneous size, an interconnective porous structure in the micrometer range, and a spherical shape. Because of this spherical shape, Bonelike[®] pellets were easier to use than polygonal granules because of their improved injectability, which may constitute an important advantage in difficult surgical approaches or in closed surgeries.

It is known that a synthetic bone graft must feature porous connectivity to ensure progressive angiogenesis and bone ingrowth, which requires macropores with a diameter

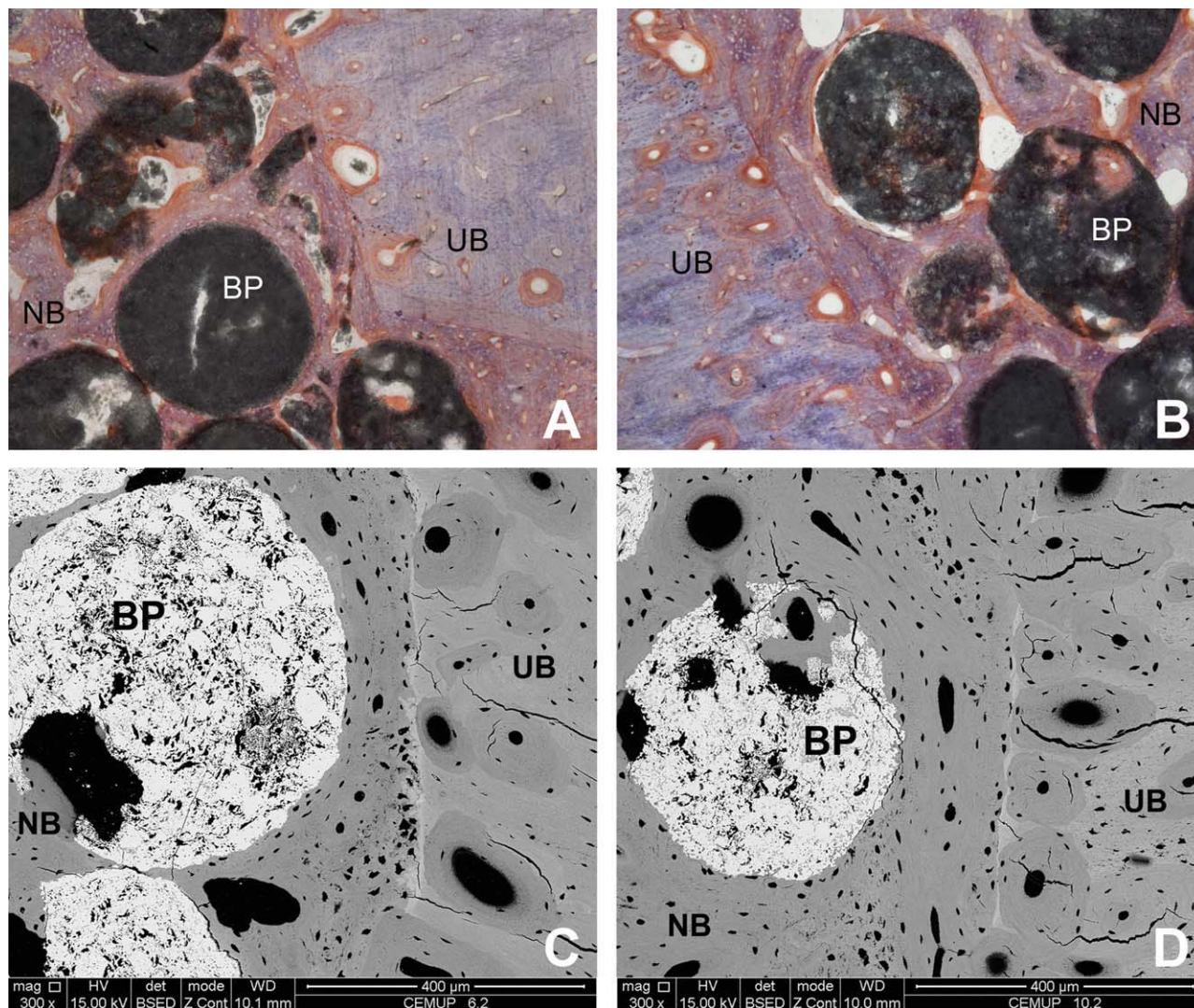


FIGURE 6. Histological (Solochrome cyanine R, magnification $\times 40$) (A, B) and SEM images (C, D) at different implantation times: 8 weeks (A, C) and 12 weeks (B, D) (NB = new bone; UB = unoperated mature bone; BP = Bonelike[®] pellet). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

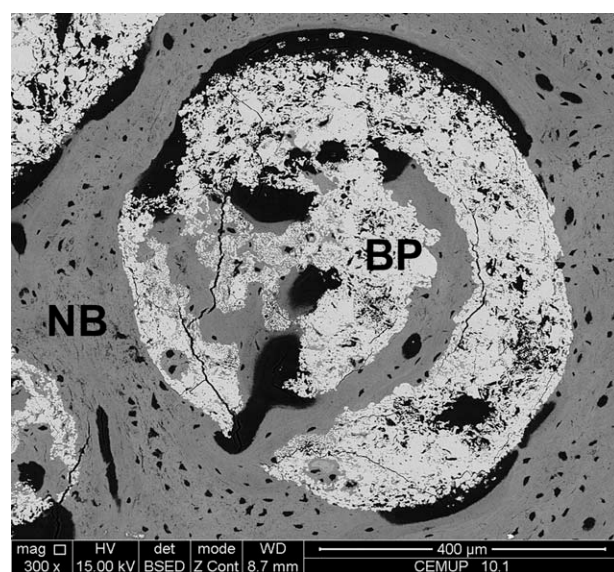


FIGURE 7. Degradation of Bonelike[®] pellets observed by SEM after 12 weeks of implantation (NB = new bone; BP = Bonelike[®] pellet).

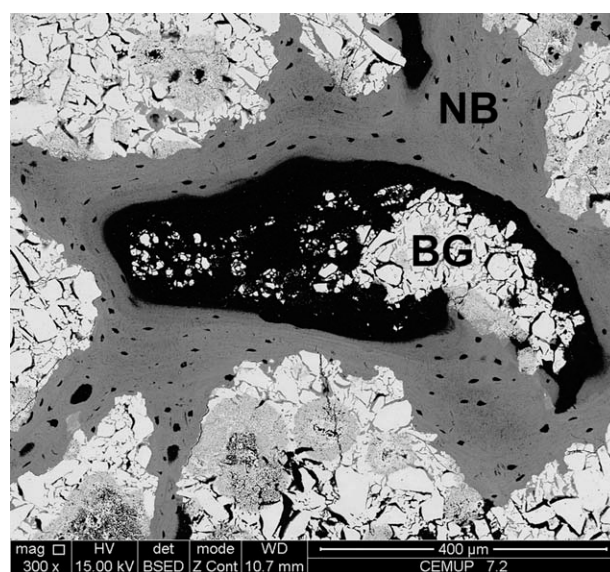


FIGURE 8. SEM image of Bonelike[®] polygonal granules after 8 weeks of implantation (NB = new bone; BG = Bonelike[®] granule).

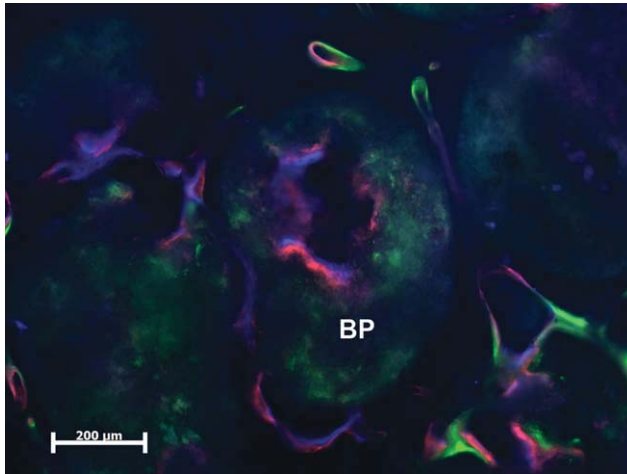


FIGURE 9. Fluorochromes labeled bone demonstrating deposited bone around and inside Bonelike[®] pellets after 8 weeks. (BP = Bonelike[®] pellet; Calcein green = green; Alizarin red = red; Oxytetracycline = blue). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

above 100 μm and micropores larger than 5 μm .^{41–44} Bonelike[®] pellets presented micropores with diameters smaller than 10 μm , and interporosity with pore diameters smaller than 343 μm , which resulted in a global porosity of 55.1%. Additionally, apparent and theoretical densities (2.98 g/mL and 3.07 g/mL, respectively) were very similar, suggesting that the pores created by the burning of the cellulose microcrystalline were mainly open (intraporosity). Microporosity increases the specific surface, which may facilitate the release of ions into the surrounding environment. This could also enable proteins and growth factors to adsorb. In granular materials, macropores are primarily formed by intergranular spaces. Therefore, and considering the value obtained for bulk density (1.34 g/mL), the spherical shape contributes to formation of macropores as pellets packaging leaves open spaces (interporosity). This aspect may facilitate cell penetration and adhesion, resulting in bone tissue growth within the biomaterials and subsequent osteointegration.

However, several studies^{45–47} have shown that pore size is not the only critical factor for osteointegration, but that in fact it is the pore interconnection size, which is often related to pore size and to the extent of porosity. Any new bone formation must always be preceded by the formation of a vascular network, which is strongly influenced by the degree of structural interconnectivity between pores. Nevertheless, in the case of resorbable bioceramics

such as β -TCP, pore connectivity and porosity volume do not seem to be such critical factors as the resorption exhibited by these materials opens up the structure.^{45,47,48} Thus, the optimal connectivity and porosity of resorbable scaffolds may be much lower than those established for nonresorbable materials.

The ovine lateral femoral diaphysis was selected because it presents a large surface of load-bearing cortical bone, which made possible the drilling of five defects without compromising femur strength. These noncritical size defects were used to ensure fast and reliable bone healing, to achieve an advanced stage of biomaterial replacement within the observation periods, and to aggrandize the number of samples by ewe.

Observations performed through optical microscopy and SEM in samples collected 8 and 12 weeks after the surgeries showed an appropriate distribution of the pellets throughout the defects with interparticular spaces filled by a healthy newly formed bone. However, the same image was also observed in defects grafted with Bonelike[®] polygonal granules, suggesting that there are no significant differences between these presentations, regarding the distribution inside the defects, if peripheral blood is the chosen vehicle. Nevertheless, the defects were only grafted after the coagulation of the mixtures, ensuring an adequate dispersion of both presentations inside the blood clot. Because of the fact that Bonelike[®] pellets preserve interparticular spaces even when densely packed, we believe that this presentation can be more advantageous if more fluid vehicles are used and that may more easily overcome limitations because of an inadequate preparation of mixtures for bone grafting.

The significantly lower percentage of defect filling in negative controls compared to defects filled with both types of Bonelike[®] suggests that this ceramic contributes to the volume increase of the newly formed bone, which may decrease the postsurgical fracture risks.⁴⁰ However, the percentage area of void spaces in negative controls was significantly higher when compared to Bonelike[®] pellets ($p = 0.03$) but not when compared to Bonelike[®] polygonal granules ($p = 0.401$). This finding may be due to the great variability in the size and in the degree of porosity of the latter presentation, resulting from the crushing procedures during its production.³⁰ Furthermore, the sharp edges of Bonelike[®] polygonal granules may adversely affect osteogenesis³¹ and induce a stronger inflammatory response in the first days after implantation.⁴⁹ In a previous study with a similar model but with a larger number of tested bone defects,⁵⁰

TABLE I. Area Fractions Inside the Defects at 8 Weeks

Treatment	Unfilled Area Fraction (%)	Void Spaces Area Fraction (%)	New Bone Area Fraction (%)	Bonelike [®] Particles Area Fraction (%)
Negative control	10 (8)	20 (4)	80 (4)	n/a
Bonelike [®] polygonal granules	1 (1)*	18 (5)	60 (5)*	22 (6)
Bonelike [®] pellets	3 (4)*	15 (3)*	58 (4)*	27 (6)

The unfilled area fraction was calculated independently from other fractions. Values represent mean (SD).

* Significantly different from negative controls ($p < 0.05$).

we obtained an $18.3 \pm 15\%$ ($p < 0.05$) reduction in the volume of Bonelike[®] polygonal granules between 8 and 12 weeks. In this study, the absence of statistically significant differences between the tested biomaterials after 8 weeks of implantation augurs a comparable *in vivo* performance for Bonelike[®] pellets. The extensive osteointegration of Bonelike[®] pellets suggests that a suitable balance between the more stable phase (HA) and the more soluble phases (α - and β -TCP) was achieved, as it allowed resorption simultaneously with bone regeneration, similarly to what happened with Bonelike[®] polygonal granules.

One of the most noticeable differences observed between defects treated with Bonelike[®] granules and Bonelike[®] pellets was the marked fragmentation of the former particles in some resorption lacunae (Figure 8). This observation suggests that some of these small particles may be either intracellularly digested or transported to lymphatic nodes by macrophage-type cells^{51,52} in the first weeks after implantation, which may constitute a potential disadvantage. This different degradation pattern may be due to the different chemical composition. Bonelike[®] can be produced either in polygonal¹¹ or spherical granules,³² and, as stated in these patents, it can be tailor-made with a specific phase composition, depending on the sintering temperature, glass amount, and composition used. Although the presence of a vitreous liquid phase during the sintering process allowed the formation and homogeneous dispersion of α - and β -TCP phases in the HA matrix for both Bonelike[®] presentations, the XRD spectrum revealed that the new production process resulted in an increase of the β -TCP fraction (19% against 7.6% in Bonelike[®] polygonal granules) and a decrease of the α -TCP fraction (13% against 24%),²⁷ while the HA fraction remained almost unaltered (68% against 68.4%). Thus, the presence of this important α -TCP fraction in Bonelike[®] polygonal granules, which is more soluble than HA and β -TCP,^{53,54} might accelerate Bonelike[®] resorption and also contribute to the extensive fragmentation of this ceramic.

The present results suggest that, albeit the referred differences, these ceramics have similar *in vivo* performance. For both ceramics, histological and SEM results revealed a very satisfactory osteointegration, signs of bone graft degradation, and simultaneous occurrence of bone ingrowth. These results are in agreement with the experimental and clinical observations made with Bonelike[®] polygonal granules^{24,27–29} and with some other calcium phosphate-based bone grafts.^{55,56}

CONCLUSIONS

Owing to its chemical composition, micropore structure, and intergranular spaces, Bonelike[®] pellets have the potential to accelerate bone regeneration. This ceramic can also be used in several surgical procedures that demand a minimally invasive approach, because of its intrinsic injectability. Further *in vivo* studies, with longer implantation periods, are under process to estimate the time needed for complete resorption of Bonelike[®] pellets.

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Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike[®]) on osteoregeneration

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**Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on
osteoregeneration**

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ABSTRACT

In the last decades, the well-known disadvantages of autografts and allografts have driven the development of synthetic bone grafts for bone regeneration. Santos *et al* developed a synthetic bone graft which consisted in the incorporation of P₂O₅-CaO glass-based system within the HA matrix (Bonelike[®]). Novel granule presentations (pellets of different sizes) were recently developed and has been tested on the ovine model. For that purpose, the physicochemical characteristics of the pellets was performed by scanning electron microscopy (SEM), mercury porosimetry, and X-ray diffraction (XRD) analysis before the *in vivo* testing. Bonelike[®] revealed no differences in chemical composition between polygonal and spherical granules. At the microscopic level, the spherical granules presented a more porous structure than the polygonal. According to mercury porosimetry analysis, BL-Spherical 500-1000 was the most porous material, followed by BL-Spherical 250-500 and by BL-Polygonal 500-1000. The spherical granules presented several advantages including easier extrusion through a syringe and better osteointegration, osteoconduction and degradation due to the presence of larger pores and a suitable particle format that can adapts to bone growth. The *in vivo* behavior of the spherical formats of granules of different sizes is very similar. The BL- Spherical 500-1000 has shown better new bone invasion and the BL- Spherical 250-500 seemed to induce a slightly faster bone regeneration, presenting less unfilled areas and less lacunae in the histological analysis.

INTRODUCTION

Bone grafting surgeries, due to disease or trauma, are performed yearly in a few million patients worldwide ¹. These procedures can be performed with the patient's own bone harvested at a different skeletal site (autograft), or with bone from donors (allograft) ². In the last decades, the well-known disadvantages of autografts and allografts have driven the development of synthetic bone grafts ³⁻⁵. Synthetic hydroxyapatite (HA), a bioactive synthetic bone graft, is the most commonly used ⁶⁻⁸ due to its osteoconductivity, biocompatibility and chemical composition similar to the bone mineral phase ^{9,10}. However, synthetic HA ⁷ is essentially non-degradable, with an annual resorption rate of 5-15% resulting in compromised bone restoration and strength ^{1,11,12}. Therefore, synthetic HA is often modified and combined with other materials for improved resorption ^{13,14}, namely, tricalcium phosphates (TCP) ¹⁵⁻²⁶.

Santos *et al* ²⁷⁻²⁹ developed a synthetic bone graft which consisted in the incorporation of P₂O₅-CaO glass-based system within the HA matrix. The resulting patented product, Bonelike[®], is composed by HA, α -TCP and β -TCP ²⁷⁻²⁹. The α - and β -TCP phases are homogeneously dispersed in the HA matrix during the sintering process, resulting in an improvement of mechanical properties and in ions incorporation in the HA matrix (e.g., magnesium, sodium and fluoride), leading to a chemical composition closer to bone mineral phase and concomitant bioactivity enhancement, as well as, controlled resorption rate ^{15,20,30-47}.

Aiming for minimally invasive application of Bonelike[®], novel granule presentations (pellets) were developed which allowed extrusion through an open bore syringe, as well as, a better adaptation to the bone defect ^{48,49}. The present study aims to validate the novel pellet presentation in a surgical animal model through all the stages of bone repair.

Physicochemical properties and respective *in vivo* performance, in non-critical size defects located in the diaphysis of ovine femurs, of different Bonelike[®] granule presentations (Bonelike polygonal 500-1000 µm, Bonelike spherical 250-500 µm and Bonelike spherical 500-1000 µm) were determined in this study.

MATERIALS AND METHODS

1.1. Bonelike[®] preparation and physicochemical characterization

1.1.1. Raw materials preparation

The preparation of phase pure HA was performed using a chemical route as described elsewhere⁵⁰. A P₂O₅–CaO based glass with the composition of 65P₂O₅–15CaO– 10CaF₂– 10Na₂O (mol %) was prepared by mixing reagent grade chemicals and obtaining a frit at 1450°C in a platinum crucible⁵⁰.

1.1.2. Polygonal granules preparation

Bonelike[®] polygonal granules were obtained by mixing of 2.5 wt% of glass with HA by a process reported previously^{29,50}. Briefly, the mixture was isostatically pressed, sintered, crushed and sieved until the 500-1000 µm particle size range was obtained.

1.1.3. Spherical granules preparation

Bonelike[®] spherical granules were prepared as described elsewhere^{29,49,50}. Briefly, Bonelike[®] was obtained by adding 2.5 wt % of glass HA and mixed with a pore forming agent. The mixture was extruded and spheronized and the pellets sintered. Standard sieving techniques were used to obtain the 250-500µm and 500-1000 µm particle size ranges.

1.1.4. Scanning electron microscopy

Bonelike[®] pellets were examined using a scanning electron microscope (SEM) equipped with an energy-dispersive X-ray spectroscopy system (FEI Quanta 400 FEG ESEM,

Hillsboro, USA; EDAX Pegasus X4M, Mahwah, USA), operating in high vacuum mode at an accelerating voltage of 15 kV.

1.1.5. Mercury porosimetry

Bulk, apparent densities and porosity were determined by mercury porosimetry (AUTO PORE IV 9500, Micromeritics, Aachen, Germany), which is based on the intrusion of mercury under pressure into the sample pores. Diameter of sample pores larger than 360 μm required a pressure between 0.5 and 33 000 psia (interporosity), while sample pores smaller than 360 μm required a pressure between 0.5 and 25 psia (intraporosity).

1.1.6. X-ray diffraction analysis

Identification and percentage quantification of Bonelike[®] crystalline phases were performed using a Siemens D5000 diffractometer with Cu-K α radiation ($\lambda=1.5418 \text{ \AA}$). The scans were performed in the range of 24– 42°, with a step size of 0.02° and a count time of 2 s/step.

1.2. Animal experimentation

Fourteen healthy, skeletally mature Merino sheep, with an average weight of 50 kg, were used. All procedures were performed with the approval of the Veterinary Authorities of Portugal (Direcção Geral de Veterinária – DGV) in accordance with the European Communities Council Directive 86/609/EEC. Surgeries were conducted under general anaesthesia. In the lateral diaphysis of each ewe's femur, a row of five circular defects with a 5.0 mm diameter was drilled through the cortex and into the medulla, using a microburr as described previously^{41,51}. Bonelike[®] was applied by extrusion through an open bore syringe, premixed with autologous peripheral blood until complete filling of the defects.

The following experimental groups were created: i) control (defect left empty); ii) BL-Spherical 250-500 (Bonelike[®] spherical granules with a 250-500 μm granulometry); iii)

BL-Spherical 500-1000 (Bonelike[®] spherical granules with a 500-1000 μm granulometry); and BL-Polygonal 500-1000 (Bonelike[®] polygonal granules with a 500-1000 μm granulometry).

Surgical follow up was performed by lateral-medial and dorso-plantar X-ray projections. Animals were sacrificed at the following implantation times: 30 days post implantation (dpi) (n=5); 60 dpi (n=3); 120 dpi (n=3) and 180 dpi (n=3).

1.3 Histological processing and histomorphometric analysis

Samples were collected and processed as described elsewhere^{41,51}. Solochrome Cyanine R and Hematoxyline Eosin stained sections were used for histological examination by light microscopy^{41,51}. Unstained sections were prepared for SEM analysis (FEI Quanta 400 FEG ESEM operating in high vacuum mode at an accelerating voltage of 15 kV) and subsequent histomorphometric determinations of bone, biomaterial, lacunae and empty spaces, using Image J software (National Institutes of Health, Maryland, U.S.A.) (**Figure 1**).

For histomorphometric analysis, several approaches were evaluated. SEM images were divided in 3 fractions for independent analysis (**Figure 1A**). A division of the defect area by the middle longitudinal axis was considered for medullar and periosteal bone bridging determination, and unfilled area determination was only considered for Fraction 1 (**Figure 1A**). Fraction 1 consists in the defect area defined as a quadrangular area with its vertices intercepting the defect wall, two periosteum and two medullar edges (**Figure 1B1**). Fraction 2 defines the medullar callus, the zone with new bone formation in the medullar region (**Figure 1B2**). Fraction 3 identifies the periosteal callus, the zone with new bone formation above the defect area (**Figure 1B3**). Percentages for new bone, Bonelike[®], unfilled area, lacunae and void spaces were determined for Fraction 1 (**Figure**

1C, 1E, 1F). The total area of faction 2 and 3 were employed to determine % of medullar callus and % of periosteal callus respectively (**Figure 1B2 and 1B3**).

The following determinations were made:

- % Bone Bridging = (no. defects with bone bridging independently of the location /total no. defects) x 100
- % Periosteal Bone Bridging = (no. defects with periosteal bone bridging /total no. defects) x 100
- % Medullar Bone Bridging = (no. defects with medullar bone bridging /total no. defects) x 100
- % New Bone = (defect new bone area/ defect total area) x 100;
- % Bonelike[®] = (defect Bonelike[®] area/ defect total area) x 100;
- % New Bone + Bonelike[®] = [(defect Bonelike[®] area + defect new bone area)/defect total area] x 100
- % Unfilled Area = (defect unfilled area/ defect total area) x 100;
- % Lacunae = (defect lacunae area/ defect total area) x 100;
- % Void Spaces = [(defect unfilled area + defect lacunae area)/ defect total area] x 100;
- % Medullar callus = (defect medullar callus area/ defect total area) x 100;
- % Periosteal callus = (defect periosteal callus area/ defect total area) x 100;

1.4 Statistical Analysis

Data was presented as mean and standard deviations (SD). One way ANOVA analysis with Bonferoni post hoc test was used to perform statistical analysis. The percentage of unfilled area, the percentage of void space, the percentage of lacunae, the percentage of new bone, the percentage of Bonelike[®], the percentage of new bone plus bonelike[®], the percentage of medullar callus, and the percentage of cortical callus were used as dependent variables.

RESULTS

2.1 Physicochemical characterization

Regarding chemical composition, XRD analysis of Bonelike® did not reveal significant differences between polygonal and spherical granules. Bonelike® BL-Polygonal granules were composed of $76\% \pm 2\%$ HA, $10\% \pm 2\%$ α -TCP and $14\% \pm 2\%$ β -TCP, whereas spherical BL-Spherical had $73\% \pm 1\%$ HA, $12\% \pm 1\%$ α -TCP and $13\% \pm 1\%$ β -TCP composition.

SEM analysis (**Figure 2**) revealed that BL-Spherical 500-1000 presented a more homogeneous size distribution when compared with the BL-polygonal 500-1000 (**Figure 2A and 2B**). At the microscopic level, the spherical granules presented a more porous structure than polygonal granules (**Figure 2C and 2D**).

When comparing the microstructure of BL-Spherical 250-500 and BL-Spherical 500-1000 (**Figure 2E-H**), differences in granule pores, both in size and in interconnectivity, were evident (**Figure 2F and 2H**). This was corroborated by the mercury porosimetry results depicted on **Table 1**. BL-Spherical 500-1000 was the most porous material (global porosity and intraporosity), followed by BL-Spherical 250-500 and by BL-Polygonal 500-1000. The same trend was observed for the medium pore diameter. Between spherical Bonelike® granules, the bulk density and average pore diameter of intrapores results showed an improved packaging for the smaller granules (**Table 1**).

2.2 Animal Experimentation

Surgical procedures were well tolerated by the animals, with no observed post-surgical complications. Macroscopic or microscopic signs of inflammation and foreign body reaction were not observed. During surgeries, the extrusion through an open bore syringe of the biomaterials mixed with autologous peripheral blood was easier when applying spherical granules (BL-Spherical 250-500 and BL-Spherical 500-1000). Polygonal granules (BL-Polygonal 500-1000) were extruded with more effort and an effect of

compaction at the end of the syringe barrel could be observed, resulting in an uneven extrusion and requiring a higher extrusion force. On X-rays, control defects were radiolucent whereas biomaterial containing defects were radiopaque due to their calcium phosphate content (data not shown). On dorso-plantar X-ray projection, some granules of biomaterial, independently of their morphology, were partially displaced into the medullar space (data not shown). Nevertheless, new bone formation around the different granules of Bonelike[®] was observed at X-rays at different implantation times (data not shown). At 30 dpi, a radiolucent peripheral circle could be observed on defects filled with biomaterial (**Figure 3A**). This radiolucency was attenuated at 60 dpi and disappeared at 120 dpi (**Figure 3B-D**). Solochrome Cyanine R sections confirmed that bone resorption on the defect borders was present at 30 and 60 dpi, whereas at 120 and 180 dpi bone resorption at the defect borders was absent (**Figure 3E-H**).

2.2.1 Histological analysis

The employed animal model displayed great variability concerning new bone formation between subjects (**Figure 4**). However, this observed behavior was persistent throughout the different sample groups implanted in the same subject. Moreover, the type of new bone observed was consistent in all implanted groups and dependent of the implantation time (**Figure 5**). At 30 dpi, some control group samples presented very small amounts of new bone (**Figure 4A**). The control groups at this implantation time showed a more immature woven and trabecular bone (**Figure 4C and 5A**). However, biomaterial group samples, especially BL-Spherical 250-500 and BL-Spherical 500-1000, contained compact new bone, thus adopting a lamellar mature configuration. Furthermore, Haversian systems can be observed with the lamellas forming Haversian canals (**Figure 5B, 5C, 6A and 6 B**). All studied Bonelike[®] granules presented good osteointegration, with new bone apposed to the granules and encircling osteocytes even for the shorter

implantation time (30 dpi) (**Figure 6A-C**). Furthermore, at 30 dpi and especially for BL-Spherical 500-1000, several intrapores could be observed (**Figure 5 and 6**). With increasing implantation times, these intrapores were filled with new bone ingrowth (**Figure 5 and 6**). This was also observed for BL-Spherical 250-500 but in a lesser extent. As implantation time increases, bone ingrowth is evident for the spherical granules, with formation of Haversian systems and lamellas encircling and closing-in on the Haversian canal, within the particles (**Figure 5 and 6**). For polygonal Bonelike[®] granules, instead of bone ingrowth, extensive fragmentation and dissolution within resorption lacunae was observe (**Figure 5 and 6**). Moreover, BL-Polygonal 500-1000 granules with sharper edges were less degraded (**Figure 6Q**).

2.2.2 Histomorphometric analysis

When compared to Bonelike[®] groups, control group samples rarely presented bone bridging on 30 dpi (38.50%). Conversely, all samples from biomaterial groups presented bone bridging during all studied implantation times. At this implantation time, 30.80% of the controls had medullar bone bridging and 30.80% periosteal bone bridging. For biomaterial samples, bone bridging was more prominent on the medullar side that on the periosteal side, with all samples showing medullar bone bridging (100%). However, periosteal bone bridging occurred on 66.67% of BL-Spherical 250-500, 88.90% of BL-Spherical 500-1000 and 66.67% of BL-Polygonal 500-1000. On 60 dpi, all samples (control and biomaterial) had bone bridging. 80% of the controls presented medullar bone bridging, while 80% presented periosteal bone bridging. All biomaterial samples had 100% medullar and periosteal bone bridging. Similarly at 120 dpi and 180 dpi, all samples presented bone bridging (100%). For 120 dpi, 85.70% of control samples had medullar bone bridging, whereas 100% had periosteal bone bridging. Control samples at 180 dpi,

had 100% medullar and periosteal bone bridging. On the other hand, biomaterial samples of 120 and 180 dpi had always 100% medullar and periosteal bone bridging.

Figures 7 and 8 depict the histomorphometric results in all studied implantation times. At 30 dpi, new bone percentage in BL-Spherical groups was tendentially higher than for control and BL-Polygonal groups but the differences were not statistically significant (**Figure 7A**). Nevertheless, when looking to effective defect filling with calcium phosphates, percentage of new bone plus Bonelike[®] (**Figure 8**), statistical significance is attained and Bonelike spherical granules achieve a more effective packing. Additionally, the unfilled area and void spaces percentages in BL-Spherical 250-500 and BL-Spherical 500-1000 was significantly lower than in the control (**Figure 7A**). At this time of implantation, Bonelike[®] groups presented a percentage of medullar callus significantly higher than the control. However, no differences were found for periosteal callus percentage.

At 60 dpi, significant differences were seen between biomaterial groups regarding new bone percentages when compared to control (**Figure 7B**). However new bone plus Bonelike[®] percentages did not display significant differences towards the control (**Figure 8**). Nevertheless, the percentage of unfilled area was null for BL-Spherical 250-500 and BL-Polygonal 500-1000, contrasting with the control and BL-Spherical 500-1000. Additionally, observed differences were related to the percentage of medullar callus in Bonelike[®] spherical granules groups that were significantly higher than the control (**Figure 7B**).

Similarly to the previous time point studied, at 120 dpi, significant differences were seen between groups regarding new bone percentages (**Figure 7B**). Yet, new bone plus Bonelike[®] percentages presented no differences towards the controls (**Figure 8**). Of note, the only group with unfilled area was the control. Therefore, and for the Bonelike[®]

groups, the only element contributing for void spaces were the lacunae (**Figure 7C**). However, there were no differences towards the control group in the unfilled area, lacunae or void spaces in the Bonelike® groups (**Figure 7C**). Only BL-Polygonal 500-1000 presented significant differences towards the control group regarding percentage of medullar and periosteal callus (**Figure 7C**).

At 180 dpi, the same significant differences were seen between groups regarding new bone percentages (**Figure 7D**). Again, new bone plus Bonelike® percentages were not different from new bone percentage on control groups (**Figure 8**). Furthermore, unfilled area percentage was absent from all groups (**Figure 7D**). However, BL-Spherical 500-1000 and BL-Polygonal 500-1000 presented a significant higher lacunae percentage and, concomitantly, void spaces percentages when compared to the control and BL-Spherical 250-500 groups (**Figure 7D**). Concerning medullar callus percentage, only BL-Polygonal 500-1000 displayed a significantly higher callus when compared to the control, whereas this group had a significantly higher periosteal callus than any other experimental group (**Figure 7D**).

Finally, new bone percentage increased in all groups in a time dependent manner (**Figure 7**). However, and regarding Bonelike® percentage, no differences were observed between groups and throughout implantation times (**Figure 7**).

DISCUSSION

Although the strong support, provided by previous studies, that the physicochemical properties of Bonelike® make it suitable for bone grafting^{35,40,41}, the development and the subsequent increase in the number of minimally invasive surgeries, namely percutaneous injection of bone substitutes⁵²⁻⁵⁴, motivated us for the need to produce Bonelike® forms with improved injectability. It should be noted that most of our studies

with Bonelike® where performed with polygonal granules^{35,40,41}, which consist in irregular fragments produced by crushing Bonelike® ceramic blocks. This heterogeneity in form may hinder bone grafting with these granules through injection devices and does not prevent their agglomeration after application. Additionally, the sharp areas of these granules may adversely affect osteogenesis⁵⁵, along with a great variability in the size and in the degree of porosity, due to the destruction of the surface layer during the crushing procedures⁵⁶. According to several authors, one feasible approach towards the improvement on injectability, is the use of spherical particles⁵⁷⁻⁶⁰, that adapt to irregular implantation sites⁶¹ preventing particle agglomeration and increasing the interporosity. Hence, it was developed a patented process, based on techniques such as extrusion and spheronization⁴⁸, to produce Bonelike® pellets with a spherical shape and a regular size. Also, due to the fact that Bonelike® pellets preserve interparticular spaces even when densely packed, we believe that this presentation can be more advantageous if more fluid vehicles are used and that may more easily overcome limitations due to an inadequate preparation of mixtures for bone grafting.

The objective of the present work was to determine the effects of glass-reinforced HA (Bonelike®) morphology on bone regeneration in a non-critical bone defect, using two different shaped granules (spherical and polygonal) and two different size ranges (250-500 µm and 500-1000 µm) at 30, 60, 120 e 180 dpi.

The controlled production of spherical calcium phosphate particles remains a challenge⁶². In the present study, a new Bonelike® granule with a spherical format was produced based on the pharmaceutical technology of extrusion and spheronization, and studied *in vivo*.

During the course of the present study the animal model displayed variability between individuals (**Figure 4**). This was more evident in control groups at 30 and 60 dpi probably

due to the non-critical size of the created defect. It was noted that some individuals presented almost no bone growth while others had new bone growth which filled the defect (**Figure 4**). Nevertheless, and as reported in literature^{51,63-66}, bone regeneration is highly dependent on factors such as age, sex, weight, breed, environmental conditions and individual variation.

Nevertheless, the biological behavior of a biomaterial is also highly affected by chemical composition and by physical proprieties ⁶⁷. The studied biomaterials had the same chemical composition, as shown by the x-ray diffraction results, despite the different production methodologies. The method employed to produce the different kind of granules tested does not influence the chemical composition of Bonelike[®]. Therefore, the observed differences in their biological behavior must be due to their morphology, macro and microstructural differences. Granule morphology, size and architecture, with the presence of macropores, micropores and interconnective pores, are physical characteristics that can affect the biological behavior of the biomaterial ⁶⁸⁻⁷⁰. Bonelike[®] spherical granules presented a more homogeneous size distribution than Bonelike[®] polygonal granules of 500-1000 μm as observed by SEM. Moreover, at the microscopic level, the spherical granules presented a more porous structure than polygonal granules which may contribute to the obtained *in vivo* results. Differences between spherical Bonelike[®] granules of 250-500 μm and 500 -1000 μm (**Figure 2**) macro and microstructures (bulk density, granule pores size and interconnectivity) were observed by SEM and corroborated with the mercury porosimetry, these differences account for the observed results *in vivo*.

As expected, defects filled with Bonelike[®] granules consisted in radiopaque circles on x-rays, which is due to Bonelike[®] chemical composition ⁷¹. The radiolucent circle observed at the periphery of the defect (**Figure 3**) at 30 and 60 dpi indicated an initial phase of

bone healing, characterized by resorption of the unoperated bone surrounding the defect⁷², as shown by Solochrome Cyanine R sections. This X-ray finding is in agreement with results of studies with zirconia-hydroxyapatite ceramic implants⁷¹. The attenuation and disappearance of the radiolucent circle at 120 and 180 dpi clearly reflects the transition of the resorption phase to the repair phase during the bone healing process⁷².

Bonelike® is a calcium phosphate synthetic bone graft with proven osteoconduction^{35,40,41,49}. This feature was also quite evident in the present study since even in subjects were controls showed little bone growth, the defects filled with biomaterial presented new bone development that resulted in bone bridging (**Figure 4**). It should be referred that at 30 dpi, and contrasting with the controls where 61.5% of the samples showed no bone bridging, biomaterial filled defects presented 100% of medullar bone bridging and periosteal bone bridging ranging from 67% to 89%, depending on the morphology of the granules. The granules with higher porosity (**Table 1**) presented an enhanced periosteal bone bridging, probably due to better cell attachment and protein absorption by biomaterial pores and a more effective vascular network ingrowth, with consequent increase of osteoconduction⁷³. On the other hand, the highest incidence of bone bridging on the medullar side is due to the ingrowth of a rich vascular network from the marrow cavity and the exposure to bone marrow stem cells^{51,74,75}, but also to some material accumulation on that particular side.

In the present study, it was verified that defects filled with biomaterial, especially BL-Spherical 250-500 and BL- Spherical 500-1000, showed earlier modeled bone process with earlier formation of Haversian systems, when compared with the controls, indicating faster bone maturation. Higher porosity and spherical format improve osteoconduction due to a better cell anchorage and concomitant cell proliferation. Also, the augmented surface area created by the pores, increases the concentration of proteins absorbed by the

biomaterial⁷³. Additionally this type of morphology permits a faster release of Bonelike[®] ionic species that promote osteogenesis.

Bonelike[®] resorption can occur by several ways such as dissolution, cell-mediated dissolution, hydrolysis, enzymatic decomposition⁶⁸, fragmentation and bone invasion⁷⁶. BL-Spherical 500-1000 presented a particular resorption pattern, with evident bone ingrowth into the particle. Initially, there was formation of larger intrapores in the particles, but with increasing implantation time the bone ingrowth was observed in intrapores (**Figure 5 and 6**). At longer implantation times, BL-Spherical 500-1000 was almost totally invaded by bone and Haversian systems, with the lamellas encircling and closing-in Haversian canal, could be observed in the bone grown in the particle intrapores (**Figure 5 and 6**). This feature was less obvious for the BL-Spherical 250-500 and rarely seen on BL-Polygonal 500-1000 probably due to intrapore dimensions (**Table 1**). An adequate pore dimension is essential for blood vessels and cells invasion, as well as, for the right flow of proteins, nutrients and waste clearance, allowing material biodegradation and bone ingrowth within the biomaterial.

Regarding granule geometry, the spherical format is more suitable to adapt to the encircling pattern of bone formation. Inversely, the sharp edges of polygonal geometry appear to hinder bone apposition. BL-polygonal granules presented resorption and degradation by dissolution and fragmentation rather than by bone invasion and replacement. The BL-polygonal granules presented extensive fragmentation with formation of multiple small particles (**Figure 5 and 6**). Other studies have proven that extensive fragmentation of biomaterial, with formation of loose particles, is harmful to osteoblasts, especially when their size is smaller than 10 μm ^{68,77}. The mobility of biomaterial particles could negatively affect the development of blood vessels and

subsequent bone formation ⁷⁸, especially if these particles have sharp edges. In fact, particles with sharper edges were less degraded in the present study (**Figure 5Q**).

At shorter implantation times, BL-Spherical granules stimulated bone growth and a more effective defect filling with calcium phosphates, consequently improving the defect stability (**Figures 8 and 7**). This was corroborated by the higher percentages of new bone, new bone and Bonelike[®], as well as, by the lower percentages of unfilled area and void spaces. The enhanced osteoconductivity of the spherical porous morphology may be due to the more effective defect packing with a good adaptation to the defect, while providing inter and intrapores for cell growth.

On subsequent implantation times, and although bone regeneration was taking place, the percentages of new bone on Bonelike[®] filled defects was smaller than on controls. This was due to the non-critical size of the defect and due to space hindrance related to the effective space occupied by the granules. Nevertheless, the percentage of calcium phosphates, on the defect, can be considered equivalent between the experimental groups. Furthermore, when looking at the medullar callus, where space hindrance is less pronounced than in the defect area, a higher percentage was observed for spherical Bonelike[®] granules, which indicates a higher osteoconduction and hence bone regeneration. This is also corroborated for the histological finding regarding the quality of the developed bone.

During the healing process, callus resorption, to reestablish the bone's original format, is slower on samples with BL- polygonal due to the lower resorption rate showed by this material, as demonstrated by our results at 120 and 180 dpi, with a larger medullar and periosteal callus than on other groups.

As the defect used in our model had an uncritical size, bone regeneration would occur without any intervention. Therefore, at 180 dpi bone had completely recovered with an

unfilled area of 0% in all samples (including controls). Furthermore, bone lacunae reached values lower than 11% in all samples, which approaches normal values of bone porosity on cortical bone (5 - 10%)^{79,80}. In this study, the percentages of unfilled area and void space (lacunae plus unfilled area) on the defect were quantified in order to determine effective percentage of regeneration, as well as, to evaluate bone fragility. Our results suggest that, during the healing process, spherical biomaterials with smaller granulometries could enable a recovery with less unfilled areas and less lacunae i.e. with a better quality bone. This was corroborated by Solochrome Cyanine R findings discussed above.

Conclusions:

Both polygonal and spherical granules of Bonelike[®] presented osteoconductive proprieties. The spherical granules presented several advantages including easier extrusion through a syringe and improved osteointegration, osteoconduction and degradation due to the presence of larger pores and a suitable particle format that can adapts to bone growth. The *in-vivo* behavior for both granulometries of the spherical formats is very similar. The BL- Spherical 500-1000 showed better new bone invasion and the BL- Spherical 250-500 seemed to induce a slightly faster bone regeneration, presenting less unfilled areas and lacunae in the histological analysis.

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DISCLOSURE STATEMENT

Authors declare that the manuscript entitled “Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on Osteoregeneration” does not have any commercial association that might create a conflict of interest. Also, any competing financial interests, actual or potential, of each author has been appropriately disclosed.

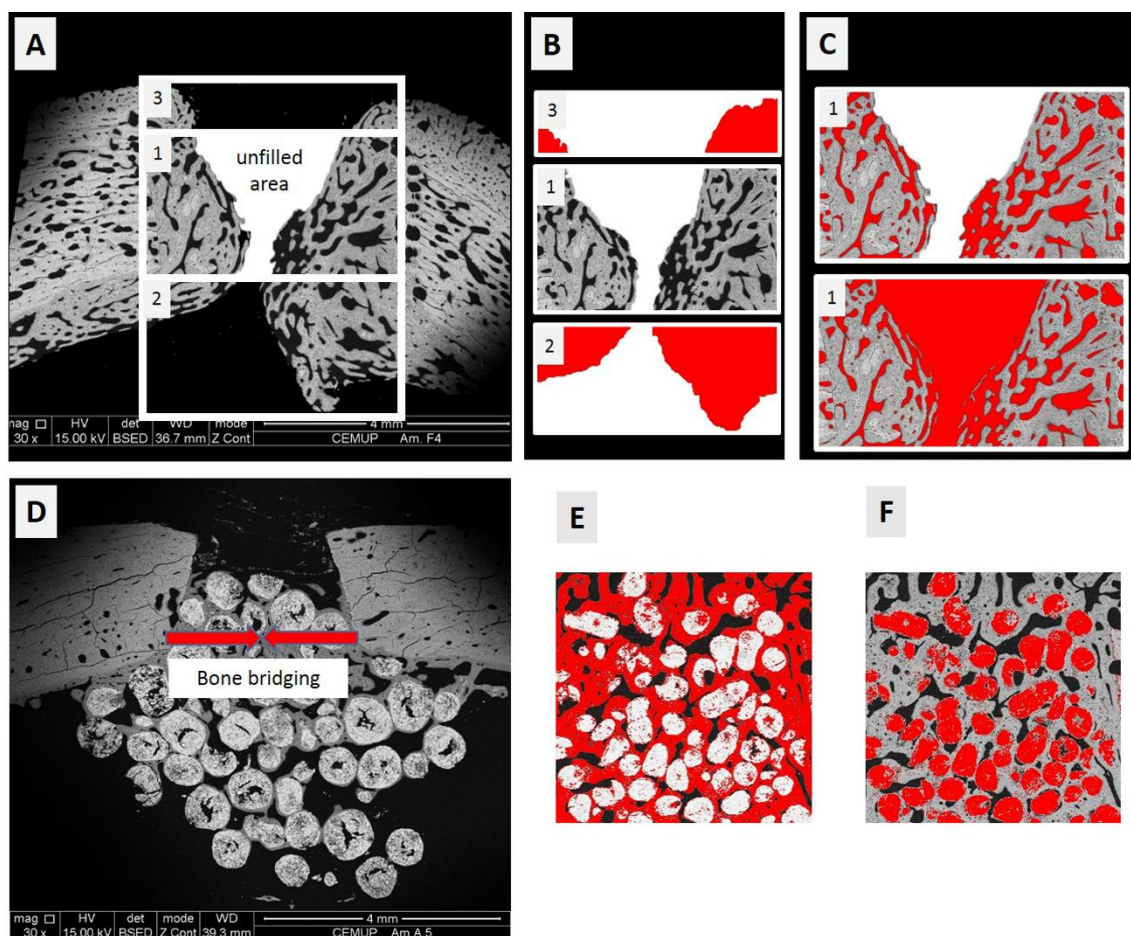


Figure 1: Histomorphometric analysis considerations. **A,B,C** - SEM 30 dpi of a control sample. **D** - SEM 30 dpi of a BL- Spherical 500-1000 sample **E,F** - SEM 30 dpi of a BL- Spherical 250-500. **A1** - Defect area fraction. **A2**-Medullar callus fraction. **A3** - Periosteal callus fraction. Red filling represents the calculated area by Image J of medullar callus (**B2**), periosteal callus (**B3**), defect lacunae area (**C1 upper image**), defect void spaces (**C1 lower image**), defect new bone area (**E**),defect Bonelike® area (**F**). Red arrows indicate bone bridging.

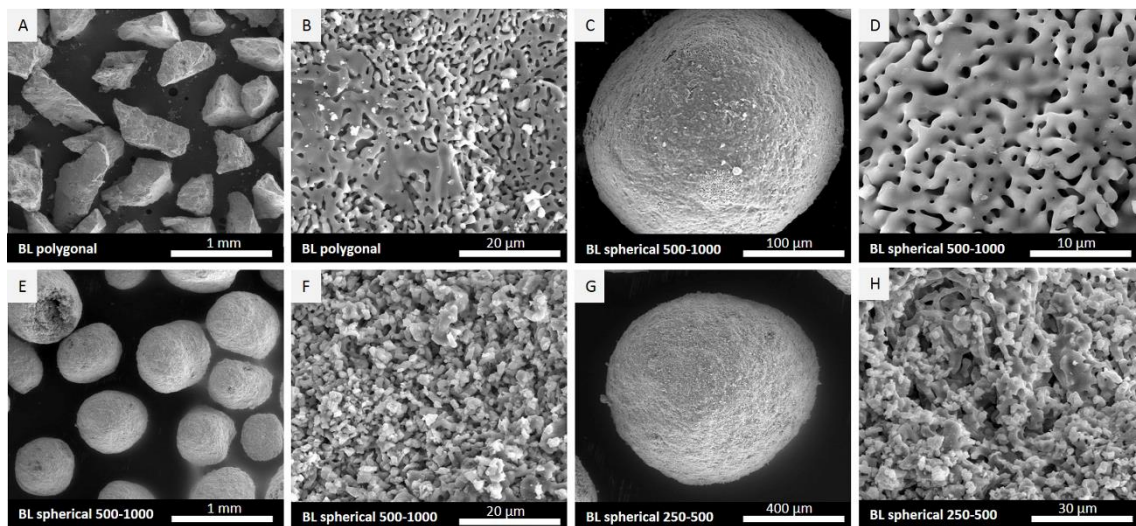


Figure 2: SEM of implanted Bonelike® granules. BL-Polygonal 500-1000 granules at distinct magnifications (**A,C**). BL- Spherical 500-1000 granules at distinct magnifications (**B,D,G,H**). BL-Spherical 250-500 at distinct magnifications (**E,F**).

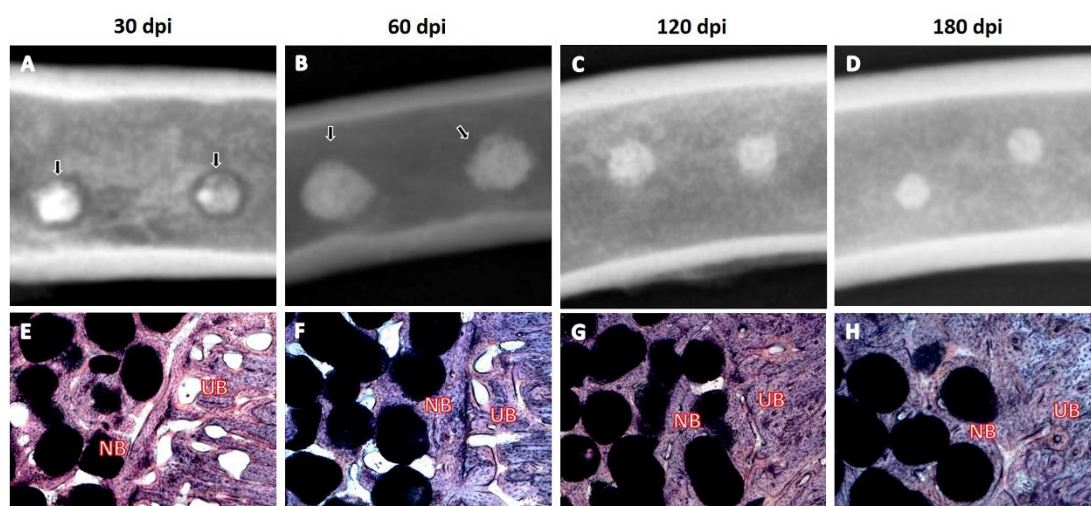


Figure 3: Bone resorption. **A** - X-rays of biomaterial filled defect. **B** - Solochrome Cyanine R sections (40x) of the defects filled with BL- Spherical 250-500. UB - unoperated bone. NB – new bone. Black arrows - radiolucency around the defect.

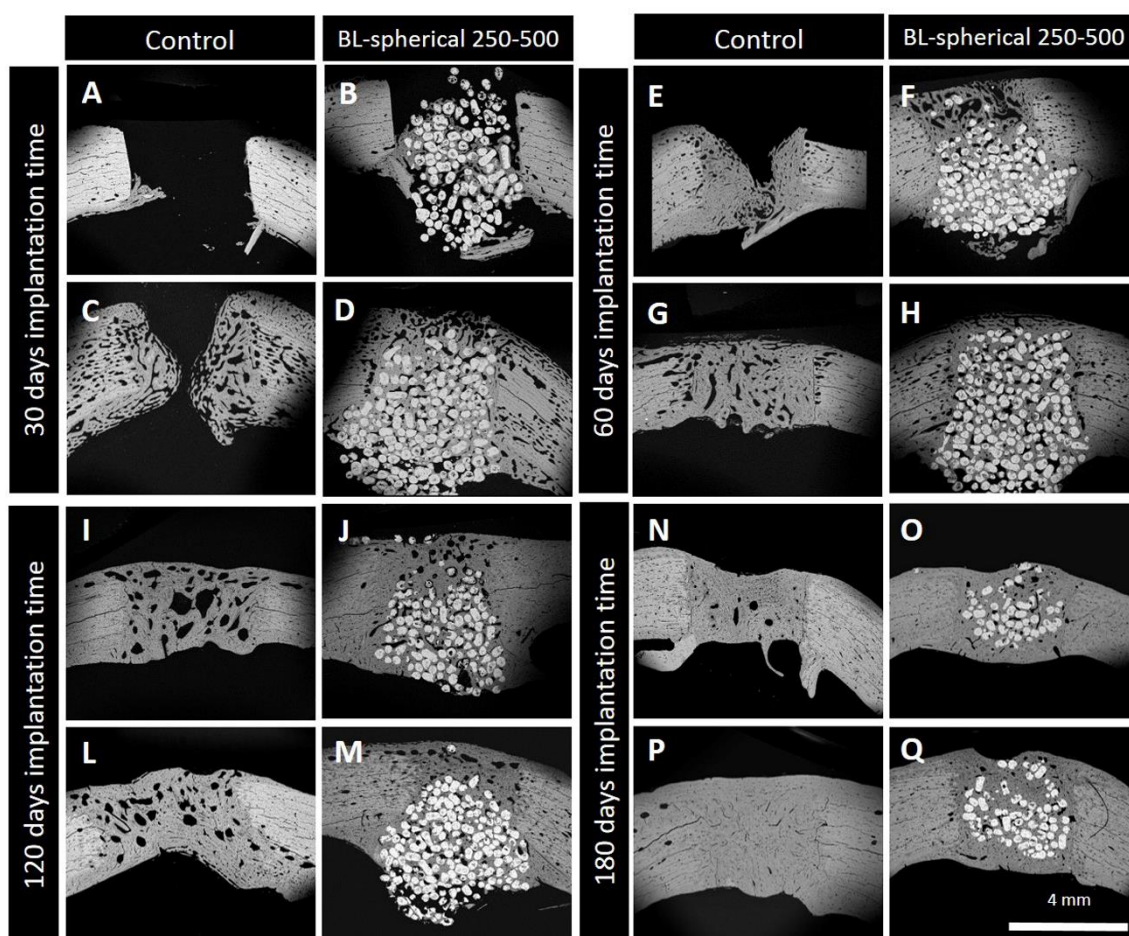


Figure 4: Variability between individuals on sheep model. SEM of control and BL-Spherical 250-500, of 2 animals from each studied implantation times.

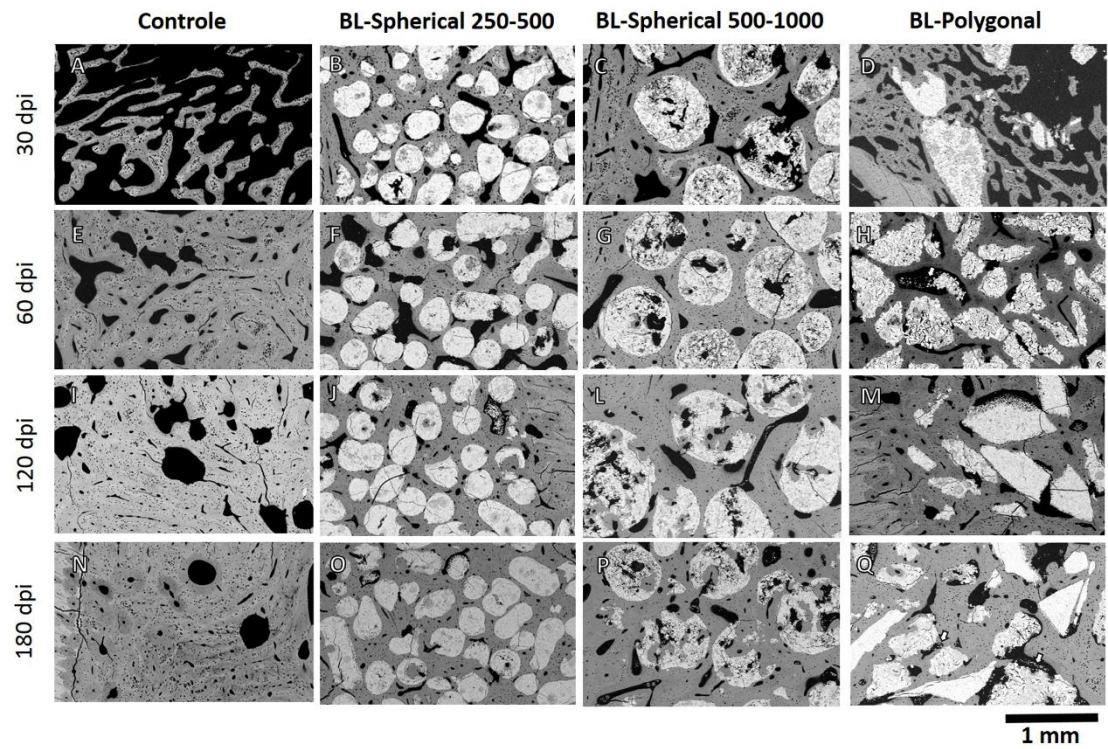


Figure 5: Healing process. SEM analysis of all groups at all implantations times. White arrow – small fragments from fragmentation of Bonelike®.

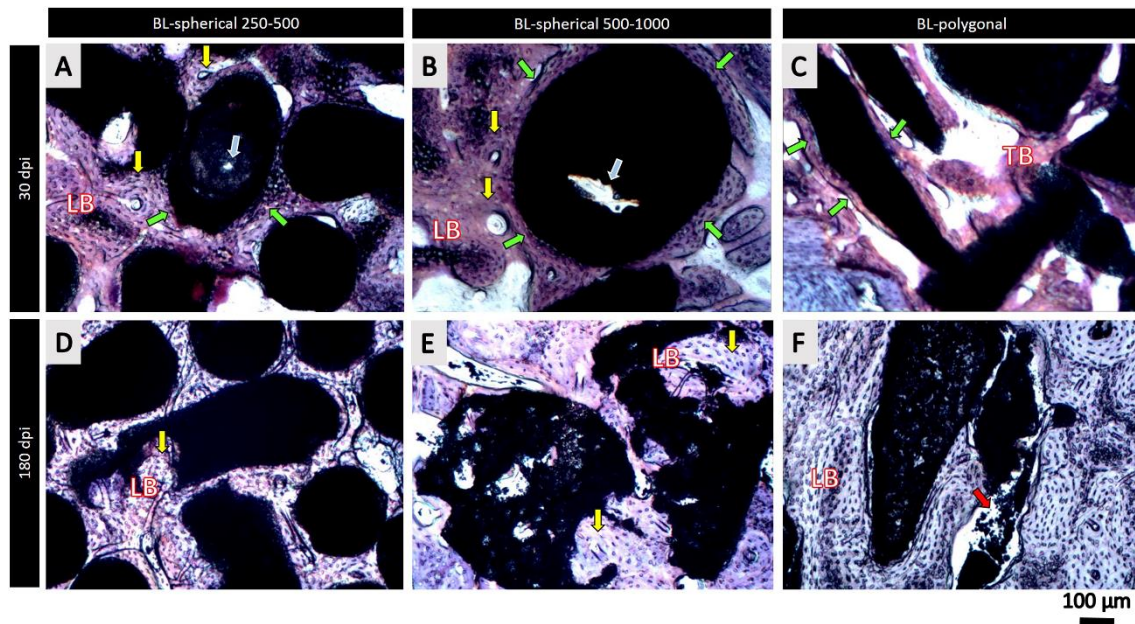


Figure 6: Bonelike behaviour at 30dpi (A,B and C) and 180 dpi (D,E and F). LB – lamellar bone; TB – trabecular bone; yellow arrow - Haversian Systems; green arrows - osteocytes organized around the periphery of the granules; Blue arrows – enlarged intrapore; red arrow – small fragments from fragmentation of Bonelike®.

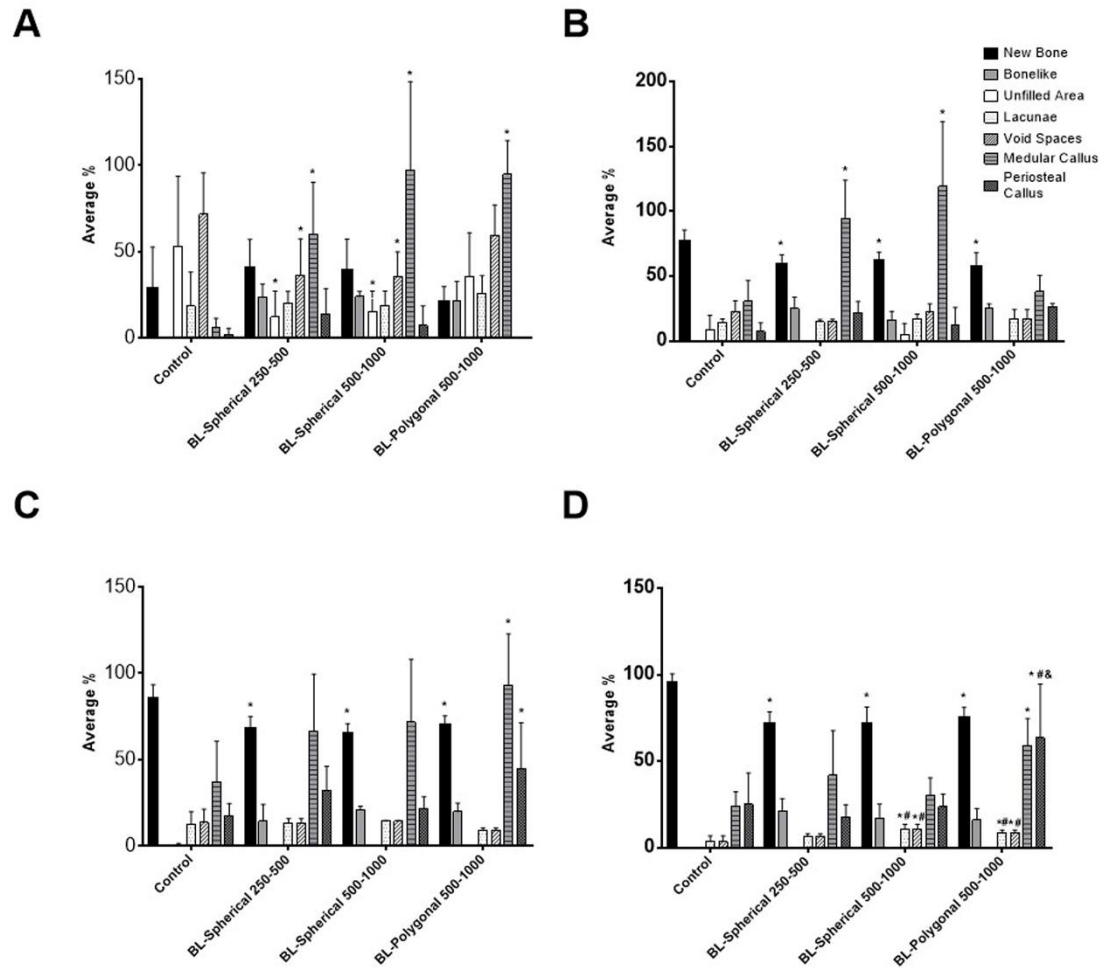


Figure 7: Histomorphometric results. **A** – 30 dpi. **B** – 60 dpi. **C** – 120 dpi. **D** – 180 dpi.

* $p < 0.05$ vs. control. # $p < 0.05$ vs. BL-Spherical 250-500. & $p < 0.05$ vs. BL-Spherical 500-1000.

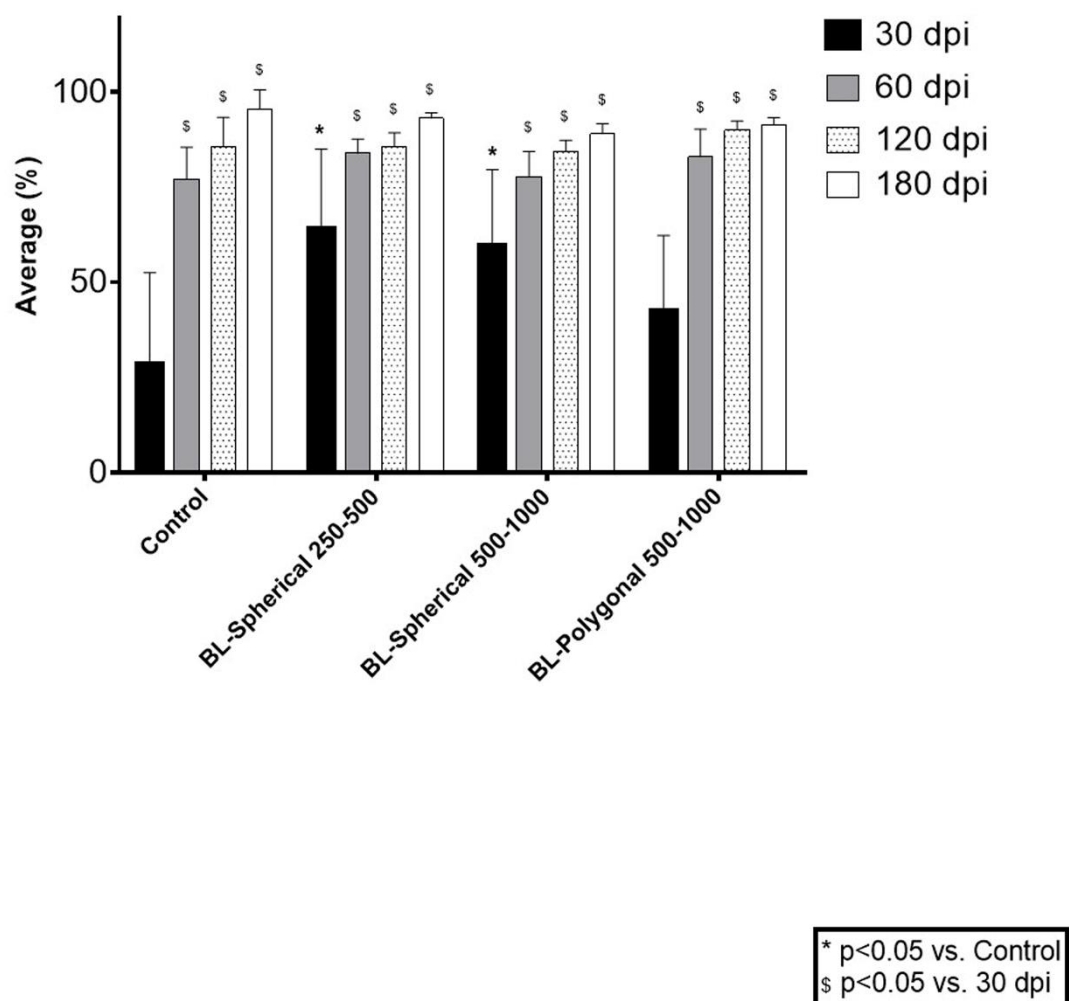


Figure 8: Percentage of New Bone plus Bonelike®. * $p < 0.05$ vs. control. \$ $p < 0.05$ vs. 30 dpi.

	Bonelike® Spherical 250-500 μm			Bonelike® Spherical 500-1000 μm			Bonelike® polygonal 500-1000 μm		
	Global	Inter	Intra	Global	Inter	Intra	Global	Inter	Intra
Hg Pressure (psia)	0.5-33000	0.5-25	25-33000	0.5-33000	0.5-25	25-33000	0.5-33000	0.5-25	25-33000
Total Intrusion Volume (ml/g)	0,33	0,2718	0,0583	0,4119	0,2319	0,18	0,2601	0,2191	0,041
Total Pore Area (m^2/g)	0,264	0,011	0,253	0,493	0,007	0,487	2,495	0,006	2,489
Average Pore Diameter (μm)	4,996	99,149	0,921	3,34	142,3	1,479	0,417	142,6567	0,0659
Porosity (%)	48,43	39,89	8,55	55,09	31,01	24,08	40,3	33,94	6,36
Bulk Density (g/ml)	1,468			1,337			1,5494		
Apparent Density (g/ml)	2,298			2,978			2,1986		

Table 1: Mercury porosimetry.

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Capítulo 5

Casos clínicos – utilização de Bonelike® em artrodeses de equinos

5 – Casos clínicos – utilização de Bonelike® em artrodeses de equinos

Neste capítulo apresentam-se os trabalhos realizados, que descrevem a aplicação do Bonelike® esférico em casos clínicos de Medicina Veterinária. Este biomaterial foi utilizado em 3 casos clínicos, onde se realizaram artrodeses da articulação interfalângica proximal, metacarpofalângica e intertársica distal e tarsometatársica.

Publicações

Artigos em Revistas Científicas e Indexadas:

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L. M. Atayde, M. G. Mendes, T. Pereira, C. Mendonça, J. Santos and A. C. Maurício (2011). Proximal interphalangeal joint arthrodesis using a bonegraft (Bonelike®) to enhance the bone fusion (clinical case). Proceedings of the 17th Congress of the Italian Association of Equine Veterinarians. Montesilvano, Italy: 289-290.

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Synthetic bone graft (Bonelike[®]) used to promote surgical arthrodesis in horses – a report of 3 clinical cases

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Abstract

Arthrodesis technique is a surgical procedure used to promote the bone fusion in the joint, resulting in immobilization. It is indicated for treatment of osteoarthritis, articular fractures, unstable joint injuries, and septic arthritis. Autologous bone graft remains the gold standard grafting substrate available in bone fusions, especially for small gaps. Significant morbidity however is associated with the harvesting of autologous bone graft and alternative bone graft substitutes have been developed. In the present work, it was tested a synthetic bone graft (Bonelike[®]) which is a glass-reinforced hydroxyapatite composite with osteoinductive and osteoconductive proprieties useful in the arthrodesis technique. Arthrodesis technique using Bonelike[®] spherical of 250-500 µm is described in 3 clinical cases. Two of the described clinical cases involving the proximal interphalangeal and the tarsal joints, presented important bone proliferation and X-ray evidence of joint fusion with good clinical prognosis. The newborn donkey metacarpophalangeal clinical case presented several post-surgery clinical complications with a negative outcome, not related with the Bonelike[®] intra-articular application. It can be concluded that Bonelike[®] granules of 250-500 µm is an appropriate synthetic bone graft substitute with osteoconductive and osteoinductive properties to be used in surgical arthrodesis, as void space filler associated with standard orthopedic procedures of stabilization of the joints, in order to promote a faster bone fusion without any local or systemic adverse reaction. This procedure improves the horse quality of life, decreasing the pain and increasing the joint stability with positive clinical outcome.

Keywords: arthrodesis, horse, joint, bone graft, bone synthetic graft, Bonelike[®].

1. Introduction

Arthrodesis of the equine joints was developed for treatment of debilitating osteoarthritis and has also been used for treatment of articular fractures, unstable joint injuries, septic arthritis, and osteochondrosis. It is a surgical procedure used to promote the fusion between opposite bones in the joint, resulting in immobilization. Because this surgical technique is used mostly in high-load low-motion joints, like the proximal interphalangeal, the intertarsal and the tarsometatarsal joint, the goal is to return the horse to athletic performance. However, the joint must be ankylosed/arthrodesed in functional alignment. Greater stability may enhance the potential for a horse to return to athletic function in a shorter period of time. Furthermore, greater stability should lead to improved post-operative comfort and less morbidity (1-4). Surgical arthrodesis involves destroying the articular cartilage of the bones to promote a bone-to-bone contact, allowing bones to fuse together. Then, the joint must be aligned and stabilized into a stable, weight-bearing position (1-4). An injection of monoiodoacetate (MIA) or ethyl alcohol into the joint could be used to destroy the joint cartilage, to perform a chemical arthrodesis. This method is an alternative to more invasive surgery and has shown evidence of improvement of the joint sintomatology. However, it produces significant short-term pain and the degree to which it results in long-term arthritic improvement and soundness varies. The use of this technique could also result in severe soft tissue damage, progression of arthritis to the adjacent joints, and persistent pain (1-5). Due to these complications, few clinicians routinely perform chemical arthrodesis (5). Usually the arthrodesis is used when the conventional medical and conservative therapeutic has failed (2, 4, 6). As it was previously referred, in the surgical arthrodesis technique, the joint cartilage is removed, in order to promote a bone-to-bone contact, and the bones must then be aligned and stabilized in a physiological position (2, 4, 7). It is described the use of bone autograft to fill the joint small gaps after removal of the cartilage (4). Autologous bone graft still remains the gold standard bone grafting substrate available, because of its osteoconductive, osteogenic and osteoinductive properties. Significant morbidity and limited amount of bone graft available however is associated with the harvesting of autologous bone graft and these disadvantages have stimulated the interest in alternative bone synthetic graft substitutes (8, 9). Synthetic hydroxyapatite (HA) is one of the most commonly used ceramics on bone grafting (10-12), due especially to its osteoconductivity, biocompatibility and stoichiometry, which is similar to mineral phase

of bone (13, 14). However, HA is essentially non-degradable, with resorption rates of only 5-15% per year (15). Consequently, the prolonged presence of HA compromise the restoration of bone strength. These limitations stimulated the development of HA forms with improved resorbability (11). Therefore, HA is often modified and combined with other materials for improved functionality and faster and more efficient resorption (16, 17). Tricalcium phosphate (TCP) is another ceramic commonly used as a synthetic bone graft. There are two crystalline phases of TCP, the alpha-phase (α -TCP) and the beta-phase (β -TCP). After implantation, the resorption rate of TCP is faster than HA (18), making TCP mechanically weaker (19, 20), but has the advantage of not remaining in the implantation site for extended periods. Santos *et al* (21-23) developed a production method which consisted in the incorporation of a glass based on the P_2O_5 -CaO- Na_2O system in the HA matrix. The final product, patented as Bonelike[®] (21-23), is composed by HA, α -TCP and β -TCP. The α - and β -TCP phases are homogeneously dispersed in the HA matrix due to the presence of a liquid phase during the sintering process, resulting in a microstructure with improved mechanical properties and enhanced bioactivity than the actual commercial HA (24-26). Other advantage of the inclusion of the referred glass during the production process is the fact that it allows the incorporation of different ions in the HA matrix, such as magnesium (Mg^{2+}), sodium (Na^{2+}) and fluoride (F^-), resulting in a chemical composition closer to the mineral phase of bone (27). The benefits of these properties have been confirmed by *in vitro* (28-31) and *in vivo* studies (27, 32, 33), along with several clinical trials (34-39). Bonelike[®] can be used in the arthrodesis technique as void filler, improving the bone contact, helping the joint stabilization and avoiding the autologous bone graft collection. Since Bonelike[®] has important osteoconductive and osteoinductive proprieties; the healing process is improved and shortened (40, 41). In the present experimental work, the arthrodesis technique using Bonelike[®] was used in 3 clinical cases: in the metacarpophalangeal joint of a newborn donkey with a severe flexural limb deformity, in the proximal interphalangeal joint of an adult horse presenting a sub-luxation of the joint, and in the distal intertarsal joint together with tarsometatarsal joint of an adult horse with bone spavin (osteoarthritis) of the joints.

Materials and Methods

2.1. Bonelike[®] preparation

For the raw materials production, the preparation of phase pure HA was performed using a chemical route as described elsewhere (42). A P₂O₅–CaO based glass with the composition of 65P₂O₅–15CaO– 10CaF₂–10Na₂O (mol %) was prepared by mixing reagent grade chemicals and obtaining a frit at 1450°C in a platinum crucible (42). Bonelike[®] spherical granules were prepared as described elsewhere (23, 40-43). Briefly, Bonelike[®] was obtained by adding 2.5 wt % of glass HA and mixed with a pore forming agent. The mixture was extruded and spheronized and the pellets sintered standard sieving techniques were used to obtain the 250-500 µm particle size ranges.

2.2. Bonelike[®] physicochemical characterization

2.2.1. Scanning electron microscopy (SEM)

Bonelike[®] pellets were examined using a scanning electron microscope (SEM) equipped with an energy-dispersive X-ray spectroscopy system (FEI Quanta 400 FEG ESEM, Hillsboro, USA; EDAX Pegasus X4M, Mahwah, USA) operating in high vacuum mode at an accelerating voltage of 15 kV. SEM analysis revealed that Bonelike[®] spherical granules of 250-500 µm presented a homogeneous size distribution and a porous structure (Figure 1).

2.2.2. Mercury porosimetry

Bulk, apparent densities and porosity were determined by mercury porosimetry (AUTO PORE IV 9500, Micromeritics, Aachen, Germany), which is based on the intrusion of mercury under pressure into the sample pores. Diameter of sample pores larger than 360 µm required a pressure between 0.5 and 33 000 psia (interporosity). Sample pores smaller than 360 µm required a pressure between 0.5 and 25 psia (intraporosity). The Bonelike[®] spherical granules of 250-500 µm presented a global porosity of 48.43%, an intraporosity of 8.55%, a bulk density of 1.468 g/ml, and an average global pore diameter of 4.996 µm (40).

2.2.3. X-ray diffraction analysis

Identification and percentage quantification of Bonelike[®] crystalline phases were performed using a Siemens D5000 diffractometer with Cu-K α radiation ($\lambda=1.5418$ Å).

The scans were performed in the range of 24°–42°, with a step size of 0.02° and a count time of 2 seconds / step. XRD analysis of Bonelike® spherical granules of 250-500 µm were composed by 73% ± 1% HA, 12% ± 1% α-TCP and 13% ± 1% β-TCP (40).

2.3. Clinical cases and surgical technique

All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC), and the NIH guidelines for the care and use of animals have been observed. A written consent from the owners was considered for the treated animals.

All surgeries were made under general inhalant anesthesia. The animals were pre-medicated with detomidine (15µg/kg) and guaifenesin (50mg/kg), induced with ketamine (2mg/kg) and maintained with isoflurane 2%.

The arthrodesis technique using the synthetic bone graft Bonelike® was used in 3 clinical cases in different joints: one adult horse proximal inter-phalangeal joint with sub-luxation, a newborn donkey metacarpophalangeal joint with a severe flexural hind-limb deformity and in the distal inter-tarsal and tarsometatarsal joints of an adult horse with bone spavin. In all surgeries the joint space was accessed and filled with Bonelike® spherical granules 250-500 µm.

On the first 2 clinical cases after the access to the joint, the cartilage covering the bones' surface was removed, with a curette, and the space between the two bones was filled with 250-500 µm Bonelike® spherical granules mixed with autologous blood. The joints were mechanical stabilized with standard orthopedic techniques, in the metacarpophalangeal joint a plate with cortical screws was used and in the proximal interphalangeal joint three lag screws 3.2 mm were employed. In the distal inter-tarsal joint together with the tarsometatarsal joint of the third clinical case, a 3.2 drill bit was placed in both joint spaces, in three directions from a single entry point in the medial aspect of the tarsus. To improve the joint fusion, the performed drilling paths were filled with 250-500 µm Bonelike® spherical granules mixed with autologous blood collected from the jugular vein during the surgery.

2.3.1. Clinical case 1: Proximal interphalangeal joint arthrodesis

The first clinical case reported is referred to an adult horse that suffered an accident, with laceration on the medial aspect of the pastern, in the right hind-limb. The laceration was disinfected, and sutured which promoted its normal healing process. When the horse returned to its routine physical activity, it was noticed that the horse presented a continuous trembling in the region of the proximal interphalangeal joint and also was elevating the right toe, during walking. Through palpation of the pastern, it was confirmed an instability on the proximal interphalangeal joint. A radiographic examination was performed with four X-ray projections, namely: lateromedial, dorsolateral, plantar lateral, dorsolateral plantar medial and dorsoplantar projections of the pastern. It was decided to perform the right proximal interphalangeal joint surgical arthrodesis.

To approach the proximal interphalangeal joint an I-shaped incision was performed in the skin over the dorsal aspect of the joint, then a Z-incision was performed over the digital extensor tendon to expose the proximal interphalangeal joint. Using a curette, the cartilage from the top of the first and second phalange was removed and the space between the two bones' surface was filled with Bonelike[®] spherical granules of 250-500 µm mixed with autologous blood. Three lag screws 3.2 mm were placed from the first to the second phalange crossing the joint space. The head screws stayed in a shelf made previously with an osteotome (**Figure 2**). The right limb was casted with a fiberglass tape, with the limb in extension. The cast was changed 20 days after the surgery and removed after 90 days. When the cast was removed an elevation of the toe during walking was evident. To improve this, the horse was shod with a heel extension. The X-ray follow-up was performed at day 0 (before the surgery), at day 155 and at day 250 post-surgery, using two projections: lateromedial and dorsoplantar projection (**Figure 3**).

2.3.2. Clinical case 2: Metacarpophalangeal joint arthrodesis

The second clinical case reports a newborn donkey with 10 days, which was born with a severe flexor deformity of the metacarpophalangeal joint. The deformity was very severe forcing the animal to support its weight on the dorsal aspect of the fetlock (**Figure 4A and Figure 4B**). During the veterinary clinical examination, a deep ulceration of the dorsal surface of the fetlock with the exposure of the articular surface of metacarpophalangeal joint was noticed. The palpation exam showed that the superficial digital flexor tendon and the deep digital flexor tendon were very strained and it was impossible to extend and move this joint. Also the common digital extensor tendon was

displaced to the lateral surface of the metacarpus. The surgical technique performed included the tenotomy of superficial digital flexor tendon, deep digital flexor tendon and common digital extensor tendon. To stabilize the joint, a surgical arthrodesis of metacarpophalangeal joint was also performed by removing, with a curette, the cartilage from the top of the third metacarpus and from the first phalange. The joint space after the cartilage removal was filled with 250-500 µm Bonelike® spherical granules mixed with autologous blood collected from the jugular vein during the surgery. A plate with cortical screws was used to stabilize the joint in a physiological position (**Figure 5**).

2.3.3. Clinical case 3: Distal intertarsal joint together with tarsometatarsal joint arthrodesis

The third clinical case reported to a Thoroughbred horse, with chronic and intermittent lameness of the right hind-limb, with a slight biomechanical instability of the tarsal region during walking. On the physical exam, the tibial and peroneal nerves blocks were positive.

The surgical approach was performed over the medial aspect of tarsus perpendicular to the cunean tendon. The cunean tendon was transected; the distal inter-tarsal and the tarso-metatarsal joints were identified with two needles. In the space of these joints, it was placed a 3.2 drill bit in three different directions using a single entry point. The drill was continuously flushed with a sterile ionic solution to avoid necrosis of the surrounding tissues. The performed drilling paths obtained were filled with Bonelike® spherical 250-500 µm mixed with the autologous peripheral blood previously collected from the jugular vein (**Figure 6**).

3. Results and Discussion

Arthrodesis of the equine joints was developed for treatment of debilitating osteoarthritis and has also been used for the treatment of articular fractures, unstable joint injuries, septic arthritis, and osteochondrosis. For instance, joint osteoarthritis is a significant cause of loss of serviceability, and successful treatment of this debilitating disease has centered on surgical arthrodesis of the joint with success rates ranging from 46–81% to 83–95% for fore-limb and hind-limb arthrodesis, respectively (44-46).

Arthrodesis techniques include: parallel screw techniques, plate fixation techniques, and plate and screw combination techniques that might include the introduction of autologous bone graft in the joint gap to improve bone fusion. These techniques involve joint

disarticulation for removal of articular cartilage and/or to perform osteostixis on the subchondral bone plates (44-46). The technique of surgical arthrodesis is used in clinical situations where it is not possible to functionally recover the joint. This procedure allows stabilizing the joint, relieving pain and improving the horse's quality of life. In clinical cases where the proximal interphalangeal and the distal intertarsal together with tarsometatarsal joint arthrodesis is performed; allow the horse to return to its normal activity, because these are low-motion joints (44-46). Despite the metacarpophalangeal joint has important movements, the arthrodesis technique is still applied since it permits to decrease pain, to increase comfort and the use of the affected limb, thus preventing the appearance of lesions in the contra-lateral limb overhead (44-46).

In the last decades, the well-known disadvantages of autografts and allografts have driven the development of synthetic bone grafts. Synthetic hydroxyapatite (HA), a bioactive synthetic bone graft, is the most commonly used due to its osteoconductivity, biocompatibility and chemical composition similar to the bone mineral phase. However, synthetic HA is essentially non-degradable, with an annual resorption rate of 5-15% resulting in compromised bone restoration and strength (15, 40, 47). Therefore, synthetic HA is often modified and combined with other materials for improved resorption, namely, tricalcium phosphates (TCP) (40, 48).-Santos et al (21-23) developed a synthetic bone graft which consisted in the incorporation of P₂O₅-CaO glass-based system within the HA matrix. The resulting patented product, Bonelike[®], is composed by HA, α -TCP and β -TCP. The α - and β -TCP phases are homogeneously dispersed in the HA matrix during the sintering process, resulting in an improvement of mechanical properties and ion incorporation in the HA matrix (e.g., Mg²⁺, Na⁺, and F⁻), leading to a chemical composition closer to bone mineral phase and concomitant bioactivity enhancement, as well as, controlled resorption rate. Bonelike[®] has been tested for bone regeneration in pre-clinical tests, involving rabbits and sheep, and it has been used in humans, with no adverse reaction or rejection of the material detected during the healing process. There is a strong support, provided by several studies already published by our research group, that the physicochemical properties of Bonelike[®] make it suitable for bone grafting (32-34, 40, 49), so its application in arthrodesis of the joints, might promote the ossification and consolidation of the articular gap, after removal of the surface cartilage. This possibility was tested in the three clinical cases reported.

The **clinical case 1**, describes the proximal interphalangeal joint arthrodesis performed in an adult Lusitano horse that suffered an accident that resulted in the laceration of the

medial aspect of the pastern. This laceration was responsible for the damage of a medial ligament, which created articular instability of the pastern and, consequently, lameness. The decision to perform a surgical arthrodesis of the proximal interphalangeal joint was based on the joint biomechanical instability observed during the veterinary clinical examination, with radiologic signs of subluxation and of an increase in the medial joint space. According to the available bibliography, the arthrodesis technique is indicated for cases of joint instability and in this specific joint, the prognosis of the horse returning to its normal physical activity without lameness is good.

During the surgery it was confirmed that this joint was not stable and there was an increased articular gap between the first and the second phalange on the medial aspect of the joint (**Figure 2B**). On the follow-up X-rays performed after the surgery, it was noticed an important bone proliferation, with evidence of bone fusion and gradual reduction of the joint space (**Figure 3**). The joint instability was, due to the surgical arthrodesis where Bonelike® was used as bone graft to improve the articular bone fusion. The observed elevation of the toe during the horse walking was probably due to the fact that the deep digital flexor tendon was also affected but this condition was corrected with a shoe with extension on the heels.

Flexor tendon disorders are usually associated with postural and foot changes, lameness, and debility. They may be congenital and therefore identified in newborn foals or acquired at an older age. Uterine malposition, teratogenic insults (arthrogryposis), and genetic defects have been either implicated or proved to cause contracted limbs in newborn foals. Chronic pain is the most common cause of acquired tendon deformity. Pain can arise from physitis, osteochondrosis, degenerative joint disease, pedal bone fracture, or soft-tissue wounds and infection. Pain induces reflex muscle contraction with shortening of the flexor musculotendinous units (50-53). Usually the flexural deformities are corrected by medical or conservative treatment, and only unresponsive and / or severe cases are indicated for surgical treatment. Palpation and the degree of mobility of the joint are important signs to decide the type of treatment. Joints that are easy manipulated are not candidates to surgical treatment. On the other hand, animals where it is not possible to perform the manual correction of the deformity, could be candidates to a surgical approach (51). The surgery main goal is to relieve the restriction created by the tightened tendons or ligaments, and therefore these structures are transected. However, a better prognosis is achieved if only a desmotomy of the accessory ligaments of the flexor tendons is performed. The tenotomy of the involved tendons is reserved only for severe

clinical cases and leads to a poor prognostic on the future athletic use of the animal (50, 51, 53); normally, animals subjected to this surgery can only be used for light pleasure riding (50).

In clinical case 2, a severe metacarpophalangeal flexural deformity was diagnosed. Through the palpation exam it was impossible to manipulate and move the fetlock joint and it was observed that the dorsal surface of the fetlock supported the animal's weight. Due to this situation, the dorsal aspect of the fetlock presented an ulceration that communicated with the joint. Due to the signs of severity and the possibility of developing complications that could worsen the clinical situation, surgical treatment was considered as a first option (53). The normal approach is to alleviate the pressure of the tightened structures that contribute to the flexural force (50, 51, 53). In the described clinical case, both the superficial flexor tendon and the deep flexor tendon were involved in the contracture of the joint and the extensor digital tendon was displaced and also forcing the joint to adopt the flexor position. It was performed the tenotomy of the three involved tendons in order to repositioning the joint into a physiological position (**Figure 5A and Figure 5B**). Although arthrodesis is not commonly used on flexural limb deformities, it was decided to apply this technique in the described clinical case, because of the lack of structural support of the joint after the tenotomy of the three tightened tendons. This approach was already described by Whitehair, Adams (54), that performed arthrodesis in a llama, in a miniature horse, and in a miniature donkey, with severe bilateral congenital flexural deformities of the metacarpophalangeal joint. The arthrodesis of the fetlock joint does not allow the animal to return to its normal athletic activity but will increase for sure, the animal's life quality. Although the fact that the surgery went as expected, the prognosis was poor due to the severity of the flexor deformity and to the ulceration, which previously exposed the articular surface to microbial contamination and the animal did not clinically recover and so it was euthanized.

Bone spavin refers to osteoarthritis of the distal intertarsal and tarsometatarsal articulations. Lesions involve degenerative joint disease, particularly on the dorsomedial aspect of the hock with peri-articular new bone proliferation, which eventually leads to ankylosis. Lytic lesions, which are part of the degenerative joint disease complex, can occasionally be seen; animals with such lesions are difficult to treat. The most frequent causes for this clinical condition are abnormal hock conformation, excessive concussion, specific athletic events (cutting, dressage, show jumping), since these events can cause increased torsional and shear forces on the distal tarsal joints (6). The disease could be

self-limiting, ending with spontaneous ankylosis of the affected joint(s) and a return to soundness. Exercise and non-steroidal anti-inflammatory drugs are used to encourage ankylosis without surgery but this is unpredictable and may take several years (55). In the early stages, intra-articular injection of corticosteroids or sodium hyaluronate (or both) may be beneficial. Also, chemical arthrodesis has been used for treatment of horse bone spavin, but this technique could have some complication as it produces significant short-term pain and the degree to which it results in long-term arthritic improvement and soundness varies. The use of this technique could also result in severe soft tissue damage, progression of arthritis in more proximal (higher up) hock joints, and persistent pain (1-5). Due to these complications, few clinicians routinely perform chemical arthrodesis. As a matter of fact, surgical arthrodesis is always a better clinical option, accelerating ankylosis of the affected joint (5).

In clinical case 3, it was performed the arthrodesis of the distal intertarsal joint together with the tarsometatarsal joint of a Thoroughbred horse with bone spavin of the two joints. The horse showed lameness of right hind-limb with a slight biomechanical instability of the tarsal region when it was performed the physical veterinary examination and the tibial and peroneal nerves' block test was positive. The objective of drilling the joint space was to destroy the cartilage and to promote the contact between the bones that form the joint. During the post-operative healing period, where no bone substitutes are used, the drilled holes are usually filled first of all, with blood, and afterwards, with fibrous tissue, that will be substituted by new bone formation that will promote the bridging of the joint (56). The application of Bonelike® in the void space of the performed holes improved the biomechanical stability of the distal intertarsal and tarsometatarsal joint. Due to the osteoconductive proprieties of Bonelike® the bone bridging was accelerated, improving the joints' ankylosis (40). Theoretically, when performing an arthrodesis, the surgeon should remove as much joint cartilage as possible, but in this specific technique of the distal tarsal joint arthrodesis, the excessive drilling causes instability and severe pain (56). It can be argued that the limited drilling of the distal tarsal joints involved in the 3-drill-tract technique does not induce complete arthrodesis but results in multiple focal areas of arthrodesis and the biomechanic stability of the distal tarsal joints is usually observed and it is eliminated the lameness (5). Using Bonelike® as a bone substitute that will fill the drilled paths, these focal areas of arthrodesis are improved and reinforced. During the post-surgical follow-up, the horse clinically improved, and the lameness disappeared. The surgical arthrodesis using Bonelike® as a bone substitute to promote bone fusion

permitted the horse to return the athletic activity and improved the horse quality of life, decreasing the pain and increasing the joint stability. As a matter of fact, the Bonelike[®] granules application can enhance the bone production due to osteoinductive and osteoconductive proprieties and shortening the healing period after the arthrodesis, promoting the joint fusion in a shorter period. As it was described in other clinical applications of Bonelike[®] in animals and humans, no local or systemic adverse reaction or rejection of the material was detected during the healing process (57).

4. Conclusions

Bonelike[®] spherical granules of 250-500 μm is an appropriate synthetic bone graft substitute with osteoconductive and osteoinductive properties to be used in surgical arthrodesis, as void space filler associated with standard orthopedic procedures of stabilization of the joints, in order to promote a faster bone fusion without any local or systemic adverse reaction. This procedure permits the horse to return the athletic performance faster, or at least improves the horse's quality of life, decreasing the pain and increasing the joint stability with positive clinical outcome.

Figures:

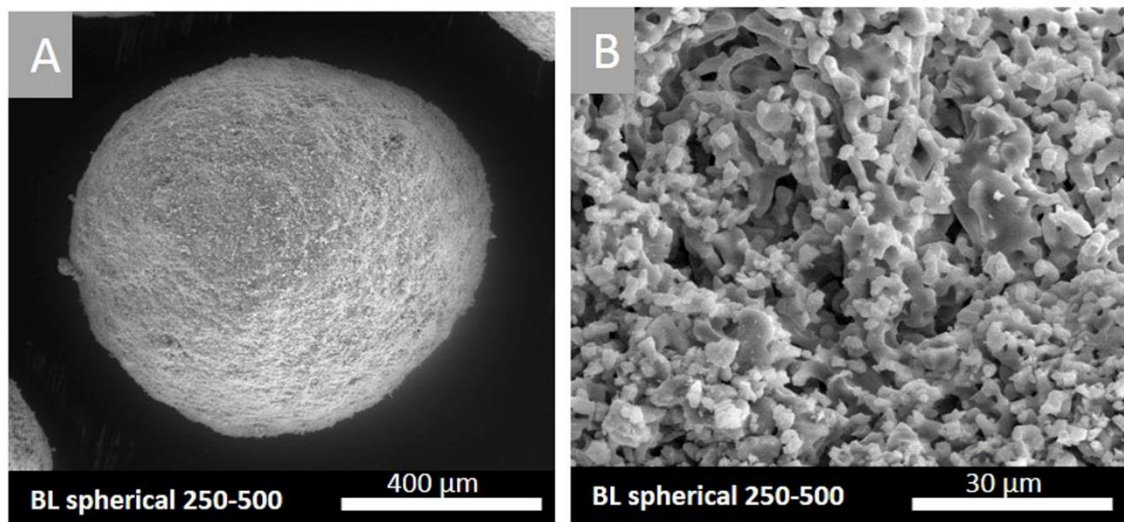


Figure 1 – SEM of Bonelike[®] spherical of 250-500 μm . Scale bar = 400 μm and original magnification of 300 x (A). Scale bar = 30 μm and original magnification of 5000 x (B).

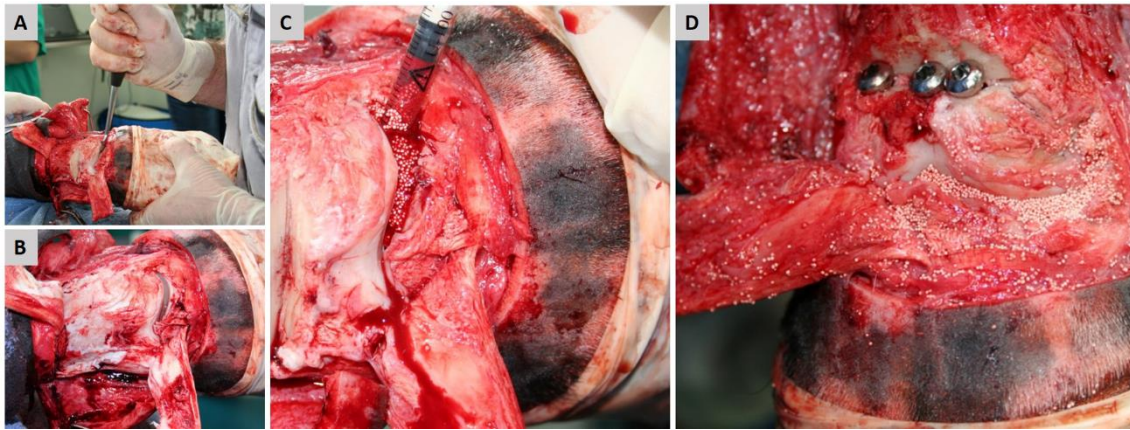


Figure 2: Proximal interphalangeal joint arthrodesis (**clinical case 1**). The cartilage from the first and second phalange was removed (**A**); Gap between the first and the second phalange can be observed on the medial aspect of the joint (**B**); The proximal interphalangeal joint space was filled with Bonelike® spherical granules of 250-500 μm mixture with autologous peripheral blood, applied with a syringe (**C**); Three lag screws were placed from the first to the second phalange crossing the joint space (**D**).

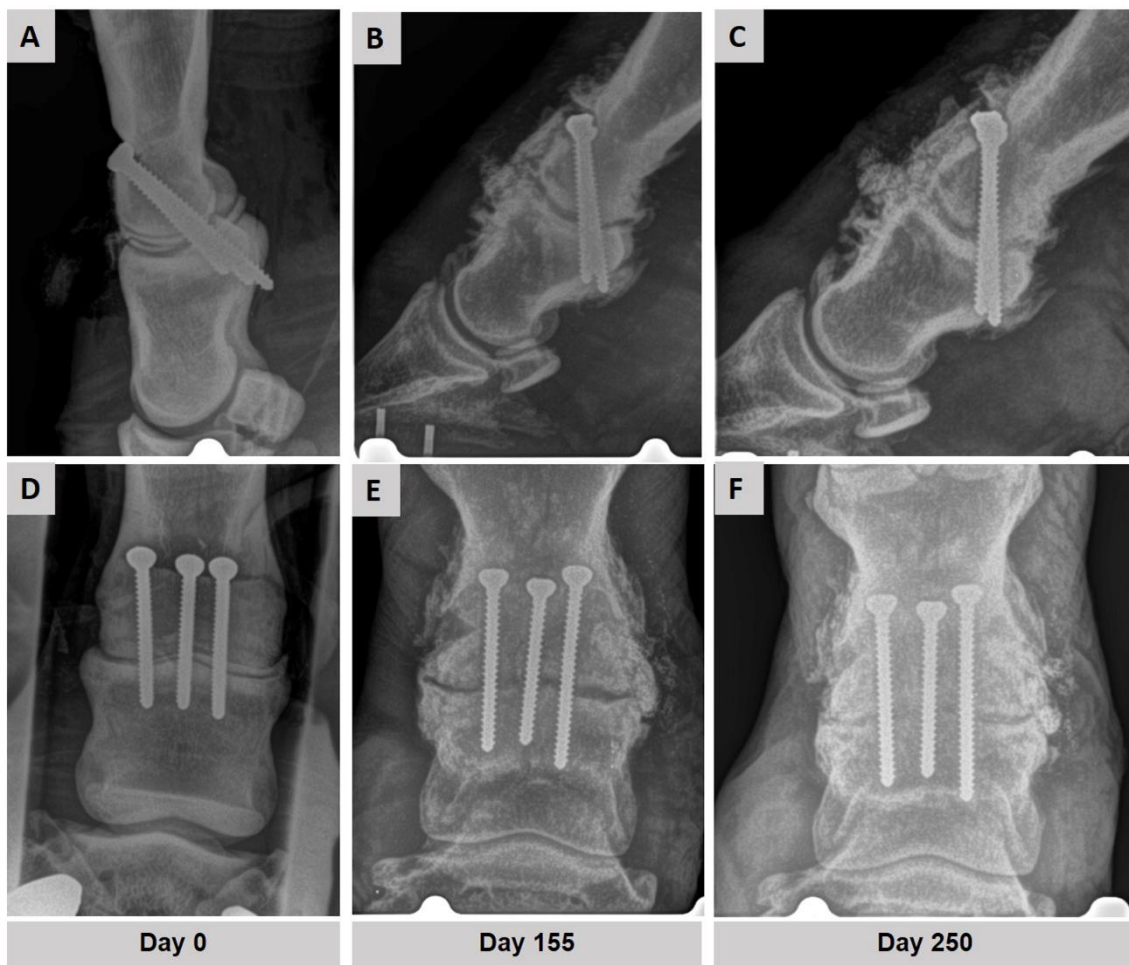


Figure 3: X-rays analysis follow-up of the proximal interphalangeal joint arthrodesis of the clinical case 1. The X-rays were taken at day 0 (pre-surgery), at day 155 and at day

250, with two projections. Lateral projection (**A, B, and C**); Dorsoproximal projection (**D, E, and F**).



Figure 4: Newborn donkey with a severe metacarpophalangeal joint flexion deformity (**clinical case 2**). The donkey supported the right hind-limb with the dorsal aspect of fetlock (**A**); Large cutaneous ulcer on dorsal aspect of fetlock (**B**).

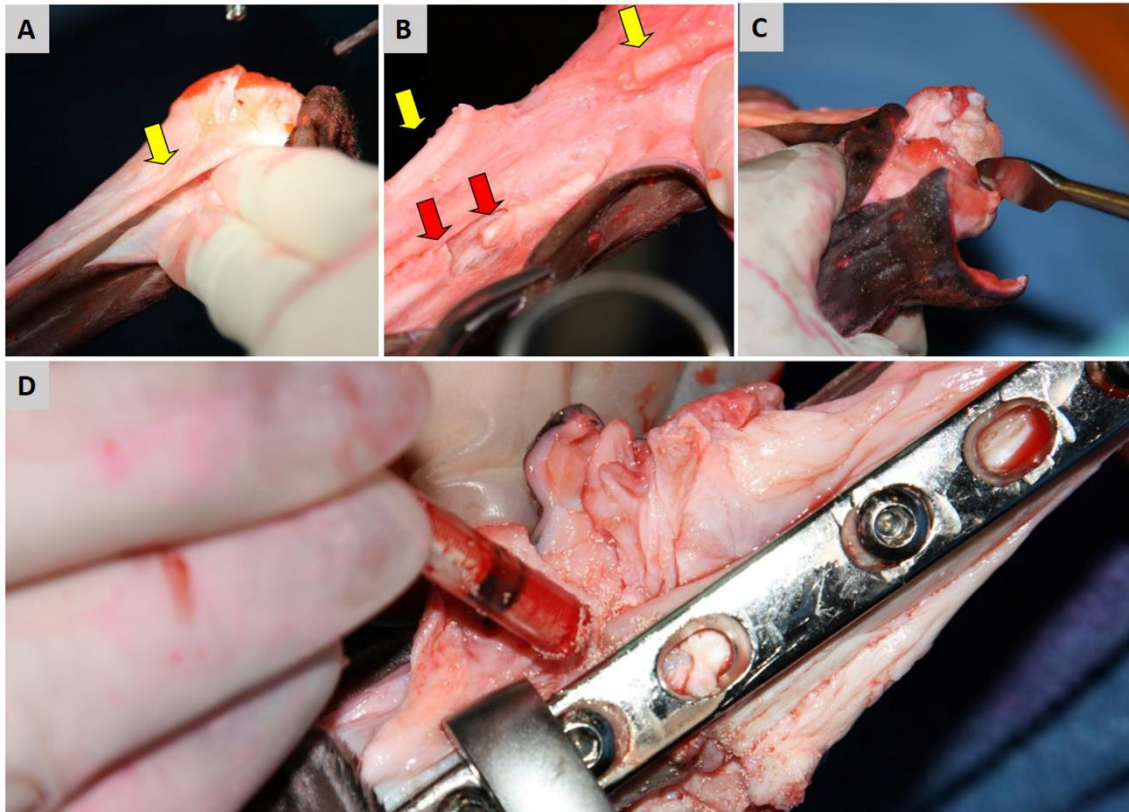


Figure 5: Metacarpophalangeal joint arthrodesis (**clinical case 2**). The extensor digital tendon (yellow arrow) was displaced caudally, and work as a flexor (**A**). Tenectomy of the extensor digital tendon (yellow arrows) and of the flexor digital tendon (red arrows) (**B**). The cartilage of the metacarpophalangeal joint was removed using a curette in order to promote a bone-to-bone contact between the metacarpal bone and the first phalange (**C**). The joint was mechanical stabilized with a plate and cortical screws and the joint space was filled with Bonelike spherical granules mixture with autologous peripheral blood, using a syringe (**D**).

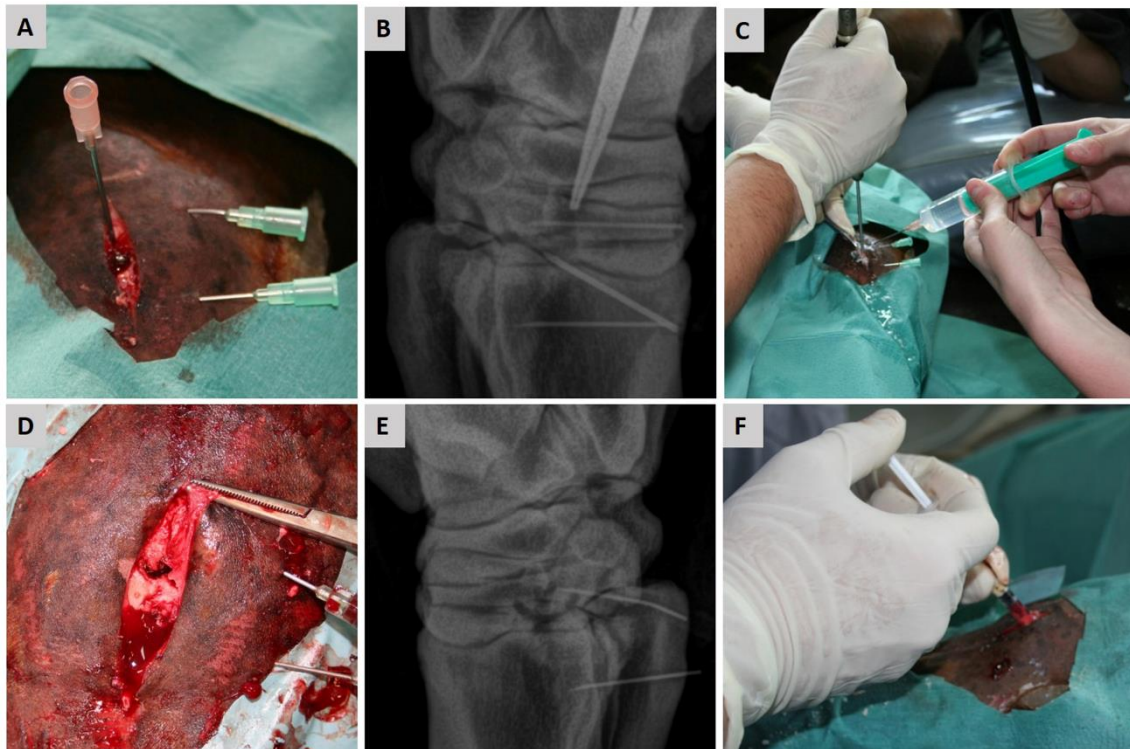


Figure 6: Distal inter-tarsal joint together with tarso-metatarsal joint arthrodesis (**clinical case 3**). A needle was placed to identify the tarso-metatarsal joint (**A**). The correct identification of the joint space was confirmed by an x-ray with a lateral projection (**B**). It was used a 3.2 drill bit to create a hole in both joints, in three different directions using a single entry point, with a continuous flushing of a sterile ionic balanced solution in order to minimize thermal damage and remove any residual bone and cartilage. (**D**). A hole in three different directions was created after drilling the joint (**E**). Lateral projection x-ray of the hole created with the drill (**F**). The hole was filled with Bonelike® spherical 250-500 μm mixed with the autologous peripheral blood previously collected.

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DISCLOSURE STATEMENT

Authors declare that the manuscript does not have any commercial association that might create a conflict of interest. Also, any competing financial interests, actual or potential, of each author has been appropriately disclosed.

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Capítulo 6

Discussão

6 – Discussão

A testagem dos biomateriais requer a escolha prévia de um modelo animal que permita comprovar a inocuidade e a eficácia do material em estudo. No início dos nossos trabalhos desenvolvemos um modelo animal, para o estudo de substitutos ósseos (Atayde, Cortez et al. 2014). A escolha do modelo ovino deveu-se às várias vantagens que este apresenta, desde a facilidade de manejo ao custo de aquisição e manutenção, peso dos animais semelhante aos humanos e a aceitação pela sociedade do uso desta espécie em experimentação animal. A preferência do modelo ovino em detrimento do modelo caprino deveu-se ao facto das maiores dificuldades de manter as cabras confinadas em jaulas individuais no período pós-operatório. Fator este de importância relevante para os nossos trabalhos, pois os animais no período pós-operatório tinham que ser confinados em jaulas individuais, para reduzir o risco da ocorrência de fraturas pós cirúrgicas (**Figura 1**).

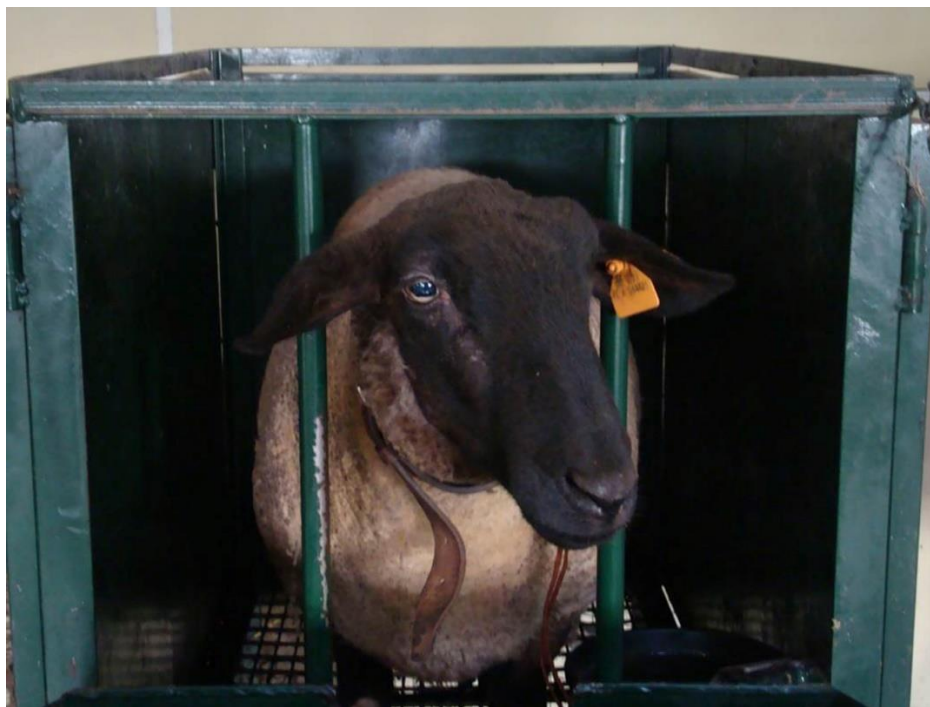


Figura 1 – Jaula individual onde as ovelhas eram mantidas no período pós- operatório.

A escolha do osso cortical em detrimento do osso esponjoso teve em conta vários fatores. O esqueleto adulto é composto por 80% de osso cortical e 20% de osso esponjoso. Além disso os ossos longos contam com uma composição de 70 a 85% de osso cortical (Seeman 2003, Clarke 2008), sendo assim, em situações traumáticas o osso cortical está mais propenso a lesionar-se. Nos estudos com osso esponjoso a quantificação da massa de osso novo no defeito, para a avaliação da performance do biomaterial, produz valores variáveis, devido à elevada atividade do osso esponjoso poder reduzir a massa de osso trabecular algum tempo após a implantação do material, independentemente da sua performance *in vivo*. Pelo contrário, a massa de osso novo cortical tem tendência a aumentar regularmente ao longo do tempo (Schopper, Ziya-Ghazvini et al. 2005). Outra desvantagem dos modelos que utilizam osso esponjoso é a escassez deste tipo de osso, que leva à dificuldade de se encontrar locais para realizar múltiplos implantes no mesmo animal em lugares semelhantes, para se poderem realizar estudos com amostras em série e testar diversos materiais (Bouré, Zeiter et al. 2008). Para ultrapassar estas desvantagens, vários estudos que utilizam o osso esponjoso limitam o número de defeitos utilizados (Gisep, Wieling et al. 2003, Harms, Helms et al. 2012), ou necessitam de realizar mais de que um acesso cirúrgico (Huffer, Benedict et al. 2007, Potes, Reis et al. 2008), com um consequente aumento do tempo de anestesia e morbilidade pós-cirúrgica. A utilização de diversos ossos para colocar os implantes também poderá ser uma alternativa, mas nestes casos a planificação do estudo tem que ter em conta as diferenças que poderão existir na estrutura óssea, na carga suportada pelos diferentes ossos utilizados e a quantidade de tecido envolvente, pois estes fatores podem influenciar o processo de cicatrização (Gisep, Wieling et al. 2003, Huffer, Benedict et al. 2007, Bouré, Zeiter et al. 2008, Potes, Reis et al. 2008, Van der Pol, Mathieu et al. 2010).

O modelo proposto e utilizado nos nossos trabalhos permite a criação de um total de 10 a 12 defeitos por ovelha, no mesmo tipo de osso com condições de carga e estrutura óssea idênticas e, além disso, com a mesma quantidade de tecido envolvente, sendo possível realizar amostras em série no mesmo animal em condições rigorosamente iguais. O procedimento cirúrgico é simples: No acesso cirúrgico só é necessário realizar a incisão cutânea e da *fascia lata*, seguido de uma disseção romba, sem riscos de lesionar qualquer estrutura

adjacente. Bouré e colaboradores também descreveram um modelo onde são criados 10 defeitos, neste caso na tíbia, mas onde o acesso cirúrgico implica a incisão no ligamento colateral e a área de trabalho está perto da articulação femorotibial, com riscos de penetração na fossa intercondiliana e possibilidade de complicações como por exemplo artrites sépticas (Bouré, Zeiter et al. 2008).

No modelo animal descrito nesta tese, optou-se pela utilização de defeitos de tamanho não crítico, para permitir a realização de testagens múltiplas e em série no mesmo animal. Na fase de desenvolvimento de um biomaterial, esta possibilidade poderá apresentar uma importância relevante, pois permite que, em condições semelhantes, sejam testadas e comparadas várias composições químicas, geometrias e tamanhos de um determinado biomaterial (Lu, Flautre et al. 1999, Boyd, Carroll et al. 2009). Além do mais, defeitos não críticos permitem observar as várias fases da cicatrização óssea em períodos de estudo curtos (Schopper, Ziya-Ghazvini et al. 2005). O tamanho do defeito de 5 mm já foi usado, por outros autores, em modelos ovinos com defeitos não críticos (Bouré, Zeiter et al. 2008, Potes, Reis et al. 2008). Estas dimensões foram escolhidas porque, apesar de apresentar um tamanho não crítico, a cicatrização demora o tempo suficiente para se poderem realizar comparações entre os comportamentos dos biomateriais estudados e os controlos (**Figura 2**). Os resultados do estudo realizado aos defeitos não preenchidos por biomaterial (defeitos controlo) confirmaram este facto, pois ao analisar-se os dados verificamos que, em todos os tempos de implantação estudados, existiram amostras, com áreas do defeito por preencher e que no tempo de implantação de 30 dias, 61.5% das amostras não realizaram pontes ósseas entre os bordos do defeito. Tal como referido anteriormente, não se optou por defeitos de tamanho maior, ou mesmo de tamanho crítico, porque não permitiriam a avaliação em série de várias amostras, tendo que se aumentar o número de animais para conseguir um universo (N) suficiente para se poder testar, com validade estatística, o biomaterial em estudo. O aumento do número de animais na experimentação animal vai contra a política dos 3Rs (*Reduction, Refinement, and Replacement*), onde se deve delinear e planificar a experimentação animal tirando a máxima informação com o uso de um número mínimo de animais (Hansen 2002). Além disso, também não podemos ignorar o facto de ao testar diferentes composições de materiais em animais destintos, confrontamo-nos

com a grande variabilidade individual que poderá existir (**Figura 3**). No delineamento da experimentação animal e na escolha do modelo, quando pretendemos inferir sobre comportamentos de materiais diferentes, deverão ser implantados, em cada indivíduo do estudo, controlos negativos (defeitos vazios) e os diversos materiais em estudo, para assim podermos controlar esta variabilidade individual. Consideramos assim que estes modelos de tamanho não crítico, com múltiplos defeitos, são muito importantes para uma primeira abordagem na testagem do biomaterial, permitindo definir a composição ideal do material em estudo e avaliando-se a biocompatibilidade do material. Escolhida a composição ideal e confirmando-se a sua biocompatibilidade, o material poderá ser utilizado nos ensaios clínicos ou, caso se ache necessário, previamente aos ensaios clínicos, recorrer-se a defeitos críticos para confirmar os resultados obtidos na primeira abordagem.

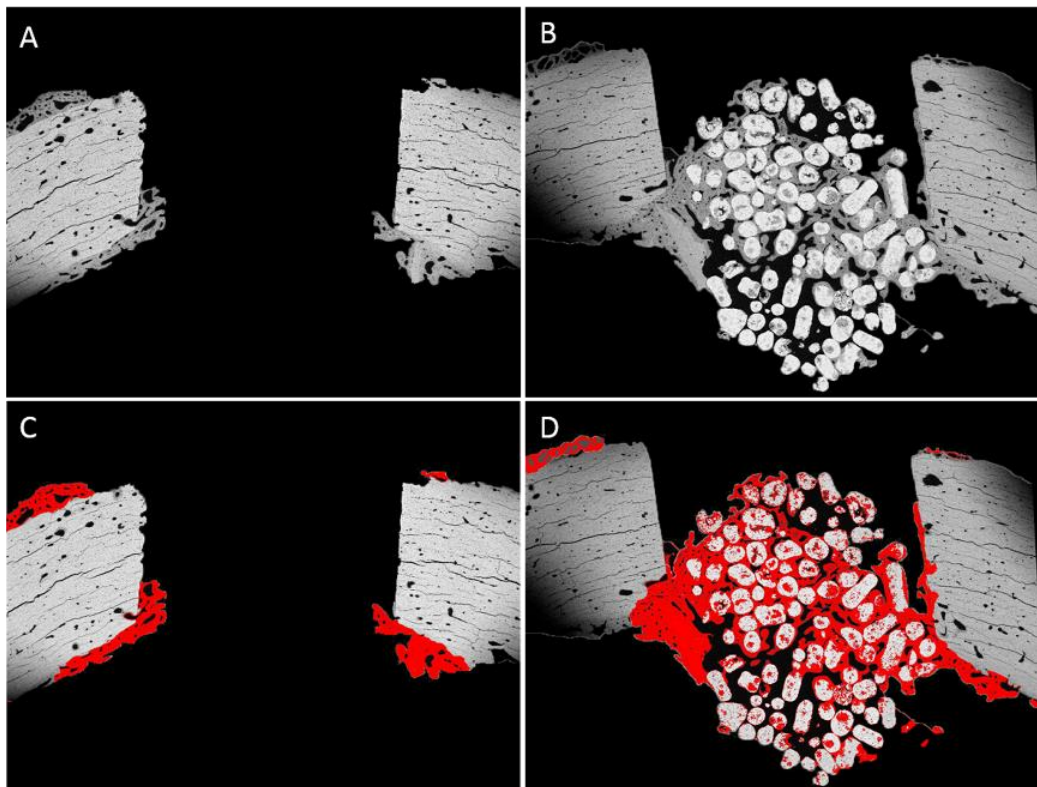


Figura 2 – Comparação do crescimento de osso novo no defeito ósseo sem biomaterial (controlo) (A e C), nos defeitos preenchidos com amostra de Bonelike® esférico de 250-500µm (B e D). Os defeitos foram realizados nos fêmures do mesmo animal. Osso novo realçado a vermelho (C e D). Imagens de SEM com ampliação de 30x com tempo de implantação de 30 dias.

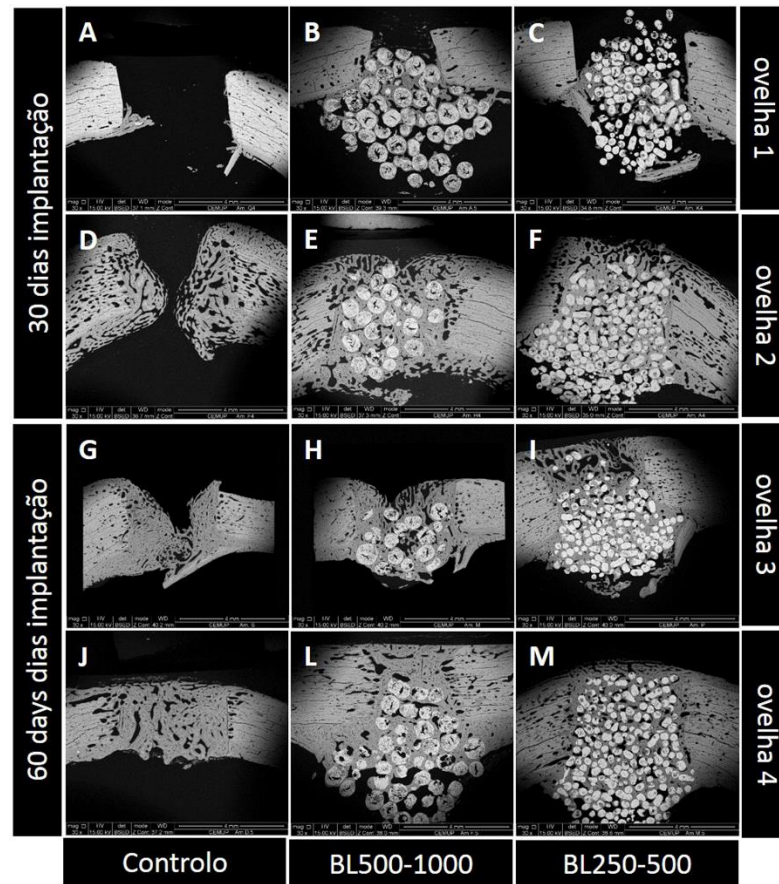


Figura 3 – Variabilidade individual. Imagens de SEM com ampliação de 30x no tempo de implantação de 30 dias (A - F); tempo de implantação 60 dias (G - M). Ovelha 1 (A - C); ovelha 2 (D - F); ovelha 3 (G - I); ovelha 4 (J - M). Controlos (A, D, G e J); Bonelike® esférico 500-1000µm (B, E, H e L); Bonelike® esférico 250-500µm (C, F, I e M).

Nestes testes *in vivo*, através de modelos animais, para a avaliação da *performance* do biomaterial, devem ser realizadas após eutanásia dos animais, análises histológicas ao tecido ósseo regenerado. No entanto, revendo a bibliografia verifica-se que muitas vezes estas análises não estão bem descritas, definidas e standardizadas e muitas delas abordam os resultados de uma forma subjetiva e descritiva sem contabilizar medições objetivas e extremamente dependentes do observador (Geuna, Gigo-Benato et al. 2004). Estas análises histológicas poderão ser descritas quantitativamente, de uma forma objetiva, através da análise histomorfometria do tecido regenerado, determinando comprimentos, perímetros e áreas (Mandarim-de-Lacerda 2003, Somanathan and Simunek 2006). Podendo calcular-se assim as áreas de preenchimento do defeito, as áreas das diferentes frações (osso, tecido conjuntivo, lacuna e biomaterial), perímetros de contacto entre o material e osso neoformado, com o objetivo de se estimar a osteointegração, osteocondução e o grau de absorção

do biomaterial (Hallman, Cederlund et al. 2001, Norton, Odell et al. 2003, Somanathan and Simunek 2006). Vários métodos poderão ser utilizados para realizar esta análise histomorfométrica. Uma das opções poderá ser delimitar os contornos da fração pretendida (Norton, Odell et al. 2003). Este método é lento e laborioso, tornando-se impossível de aplicar quando existe um grande número de amostras para analisar e/ou quando estas amostras são constituídas por múltiplas frações pequenas. Como alternativa poderão ser usadas grelhas de contagem, que consiste na colocação de uma grelha, sobreposta à imagem, que é composta por cruzes equidistantes, sendo as cruzes que tocam na fração em estudo contadas e divididas pelo total das cruzes presentes na região de interesse, para assim se poder obter a percentagem da fração em estudo (**Figura 4**) (Gundersen, Bendtsen et al. 1988, Lopes, Santos et al. 2001, Mandarin-de-Lacerda 2003, Cortez, Silva et al. 2011). Segundo Gundersen e colaboradores este método é menos trabalhoso e mais preciso do que a delimitação dos contornos da fração pretendida (Gundersen, Bendtsen et al. 1988). Outra alternativa, mais simples e rápida, apesar de ainda não ser muito utilizada, poderá ser a medição destas áreas de uma forma semiautomática, utilizando técnicas de segmentação de imagem computadorizadas.

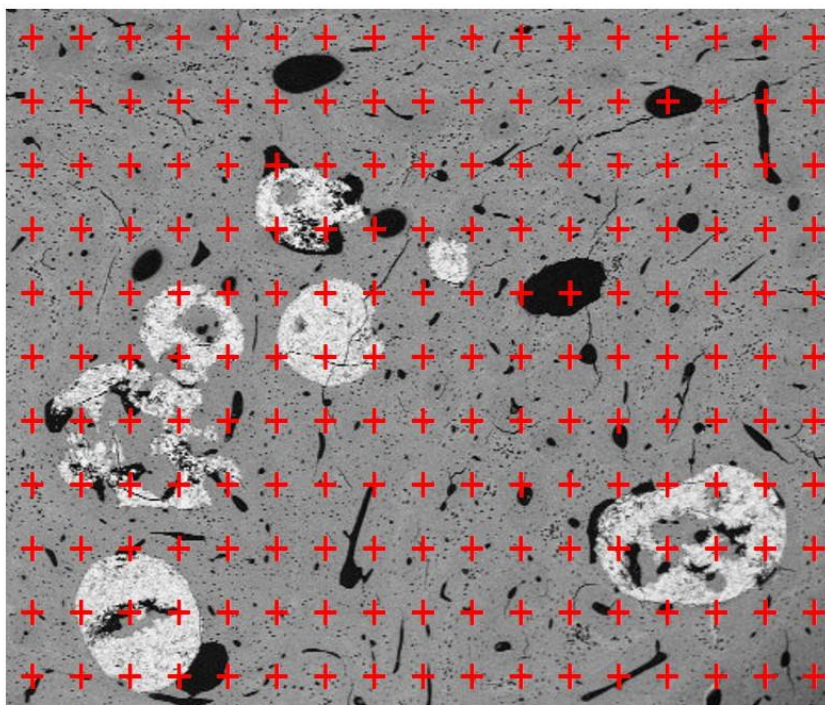


Figura 4 – Grelha de contagem.

Durante a realização deste trabalho experimental, também se pretendeu desenvolver e validar uma metodologia de segmentação de imagem (*Threshold*) para ser utilizada na análise do comportamento de biomateriais na regeneração de tecido ósseo. Para tal, foi comparado este teste de *Threshold* com o teste da grelha de contagem, utilizando amostras de defeitos ósseos implantados com Bonelike® esférico (*pellets*) de 250-500µm durante um período de implantação de 120 dias. Calcularam-se as áreas de lacunas, osso novo e biomaterial pelos dois métodos e compararam-se os resultados obtidos em cada teste (Atayde, Cortez et al. 2013).

No teste da grelha, a contagem de cada imagem foi repetida utilizando 4 grelhas, com espaçamento entre cruzes diferente, para assim calcular qual o limiar deste espaçamento, onde se obtêm resultados fiáveis. Quanto mais curto for este espaçamento mais reais serão os resultados, mas mais moroso e trabalhoso se torna o teste, pela necessidade de contar um grande número de cruzes. Na situação ideal deve-se encontrar o maior espaçamento possível, onde se contem o menor número de cruzes com resultados fiáveis. As frações em estudo (lacunas, osso novo e biomaterial) foram calculadas 5 vezes para cada espaçamento entre cruzes, com a grelha sobreposta na imagem em posições diferentes e aleatórias, para verificar a variância das contagens em cada grelha e assim se poder validar este teste (Gundersen, Bendtsen et al. 1988). Como era de esperar o coeficiente de variação baixou à medida que se diminui o espaçamento entre cruzes das grelhas. Na comparação entre o teste da grelha e o teste de *Threshold* não se verificaram variações significativas nos resultados obtidos, concluindo-se que este teste de *Threshold* poderia ser utilizado com este tipo de amostras.

Na realização de ambos os testes, contagem com grelha e *Threshold*, utilizou-se o *software* ImageJ (ImageJ 1997-2012). Na contagem com grelha utilizou-se o *plugin gird* (**Figura 5**) para sobrepor a grelha na imagem e o *plugin cell couter* para realizar a contagem das cruzes (**Figura 6**). O procedimento de contagem foi bastante trabalhoso e moroso, pois era necessário contar muitas cruzes para o cálculo de cada fração (**link pra filme** http://youtu.be/jYfN_Qm_jII).

O teste de *Threshold* mostrou-se muito mais simples e rápido de realizar (**link para filme** - <http://youtu.be/VbRp8SjzG8Y>). Este tipo de segmentação de imagem, utilizada nos nossos trabalhos, tem a desvantagem da imagem ter de

estar ou ser transformada para a escala de cinza. As imagens de SEM são ideais para realizar este tipo de análise, pois são obtidas na escala de cinza. Para a realização deste teste no imageJ, aplica-se o ajuste de imagem com a ferramenta *Threshold (Image / Adjust/ Threshold)* (**Figura 7**). O ponto crítico deste teste é a seleção dos limiares de corte para cada fração, podendo ser feito visualmente por um operador, regulando e procurando o valor de corte que apresenta resultados satisfatórios. Neste caso para diminuir a influência da subjetividade da escolha do operador, no momento da realização do teste, este não deve ter informações sobre a amostra em análise. No caso das imagens com um bom contraste, entre as diferentes frações em estudo, que originam picos distintos no histograma, pode-se encontrar um valor fixo para os limiares de corte em cada fração, no ponto médio entre cada pico (na vala do histograma) (Efford 2000, Burger and Burge 2009), ficando assim a análise imune à subjetividade da escolha do operador, com um critério semelhante em todas as amostras analisadas. Definidos os limiares de corte do *Threshold*, a fração tem que ser selecionada e medida. Esta tarefa no *software* ImageJ implica a realização de vários passos, nomeadamente: criar a seleção da fração pretendida (*Edit / Selection / Create Selection*) (**Figura 8**); depois medir a área selecionada (*Analyse / Measure*) (**Figura 9**). Para facilitar estes procedimentos, tornando a análise mais rápida, criou-se um *Macro*, que facilita e permite realizar a seleção e medição da área pretendida apenas num só passo (**Figura 10**).

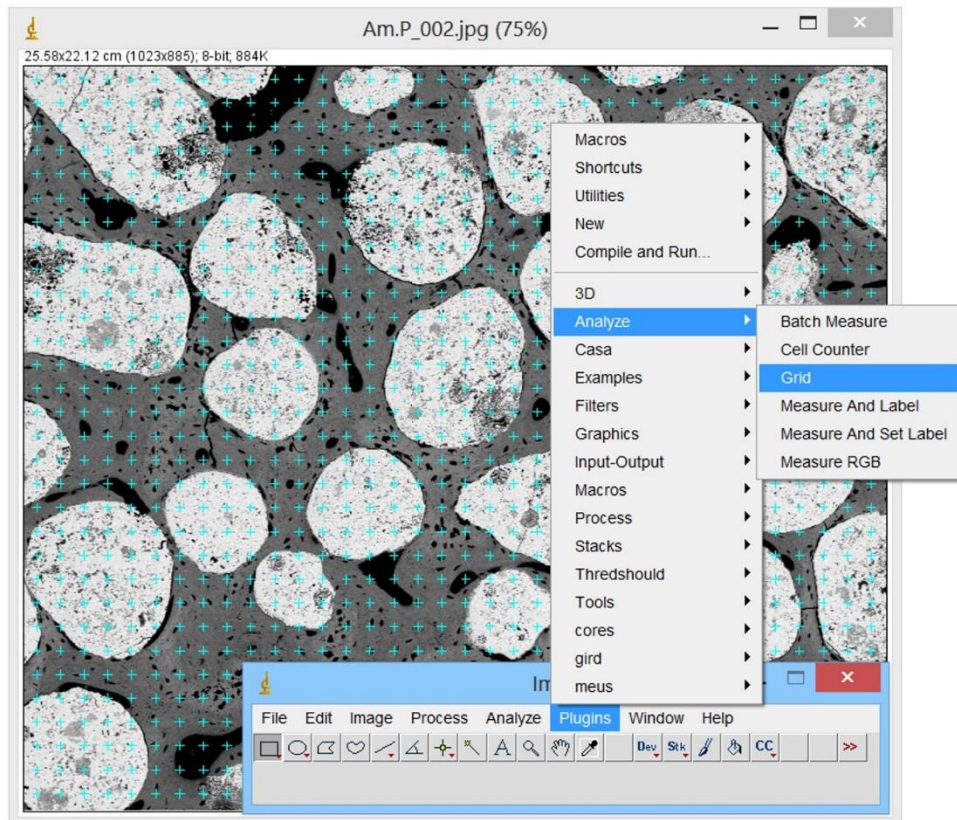


Figura 5 - Colocação da grelha sobreposta à imagem no *software* ImageJ.

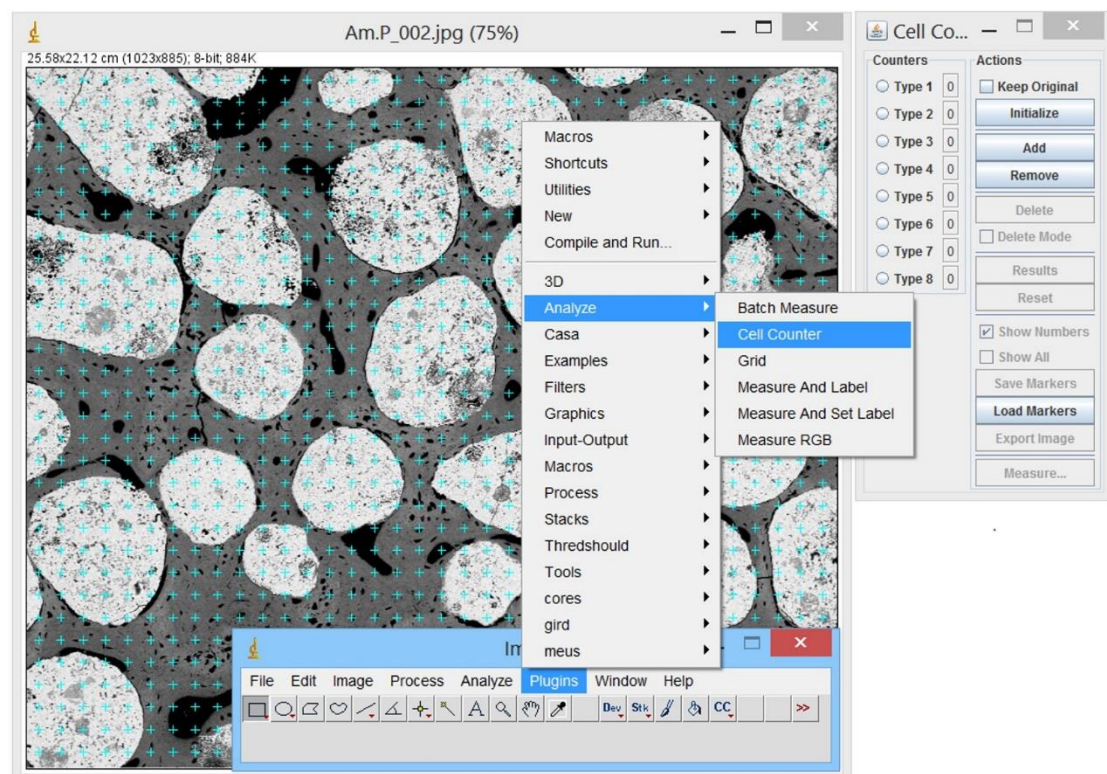


Figura 6 - Ativação do *plugin cell counter*, para contar as cruzes da fração pretendida.

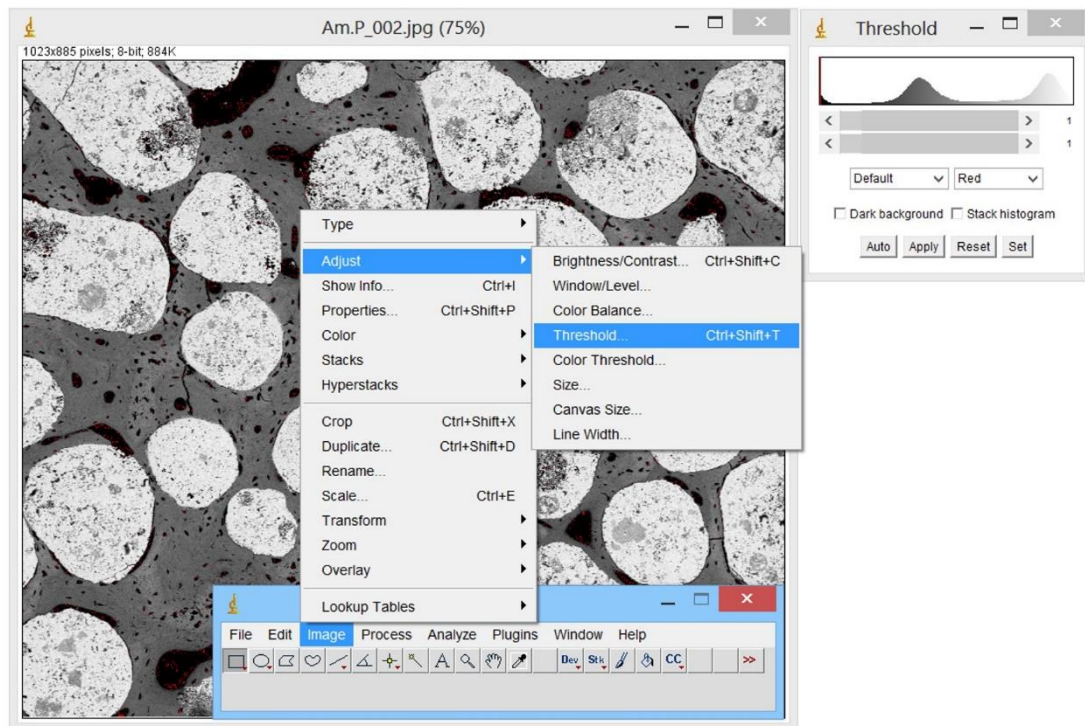


Figura 7- Ativação da ferramenta *Threshold*.

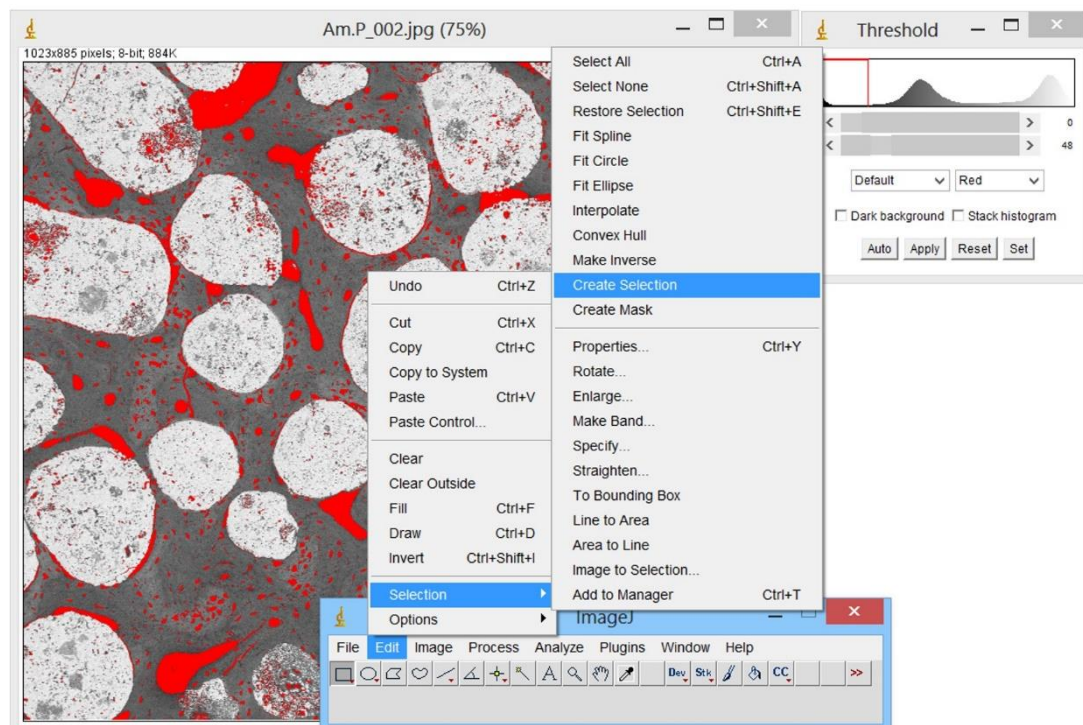


Figura 8 - Criar a seleção da fração isolada pelo método de *Threshold*.

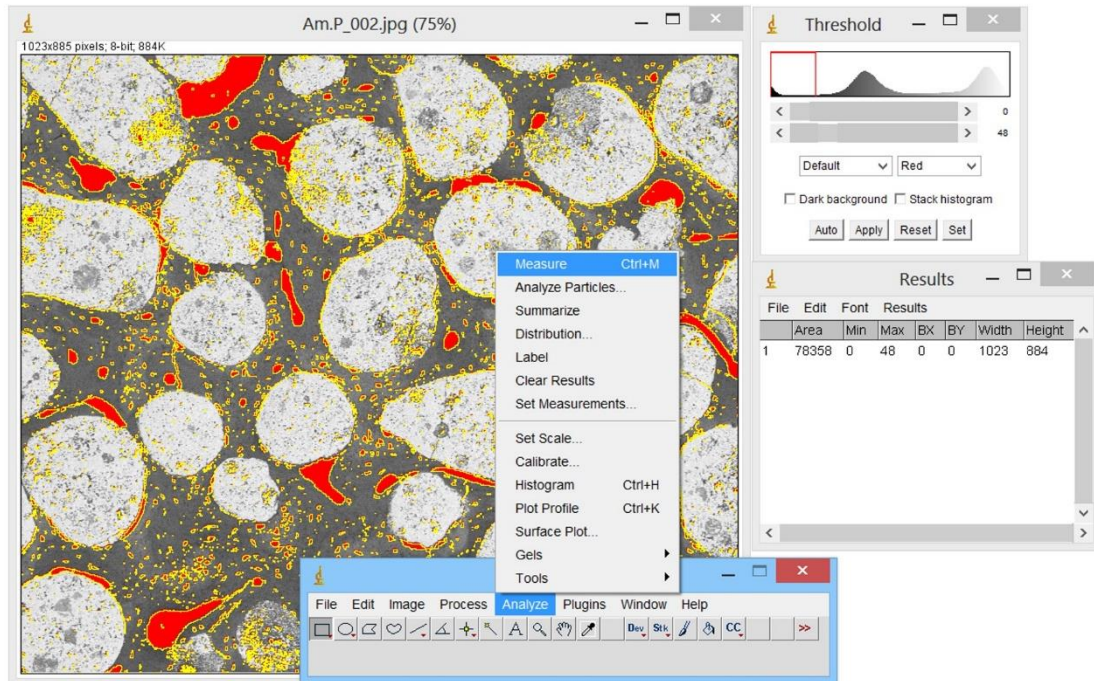


Figura 9- Medir a área selecionada.

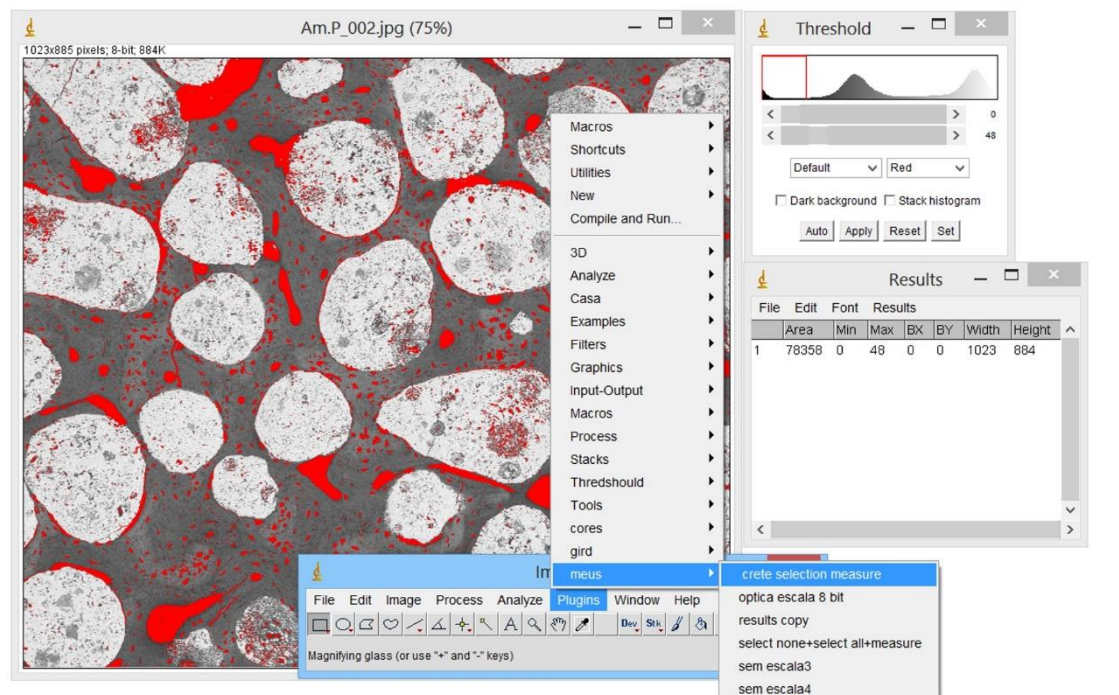


Figura 10 – Macro, desenvolvido para efetuar a seleção e a medição da fração pretendida.

Para caracterizar o processo de regeneração do tecido ósseo no modelo animal proposto, sem a influência do biomaterial, foram analisadas 29 amostras de defeitos circulares sem preenchimento (defeitos controle), realizados no fêmur de ovinos, nos tempos de implantação de 30, 60, 120 e 180 dias (Atayde, Cortez et al. 2014). Foram escolhidos estes tempos de implantação, para permitir visualizar todas as fases da regeneração óssea. De um modo geral, a regeneração óssea depende do aporte sanguíneo e da estabilidade mecânica do local da lesão (Schenk and Hunziker 1994, Griffon 2005). Em casos da existência de uma fixação rígida, com bom aporte sanguíneo e com um espaço entre fragmentos menor que 0.01 mm, verifica-se uma cicatrização primária com a formação direta de osso lamelar com uma orientação paralela ao eixo longitudinal do osso, sem formação de calo ósseo (Shapiro 1988, Mann and Payne 1989, Griffon 2005). No extremo oposto, em lesões com mobilidade e um deficiente aporte sanguíneo, a cicatrização óssea poderá ser mediada pela formação prévia de tecido cartilaginoso, sendo que, em casos extremos esta cartilagem poderá não ossificar e formar-se uma não união (Griffon 2005, Marsell and Einhorn 2011). O defeito criado no nosso modelo apresentou um padrão de cicatrização descrito por vários autores (Glimcher, Shapiro et al. 1980, Shapiro 1988, Griffon 2005, Shapiro 2008, Dwek 2010, Marsell and Einhorn 2011), para lesões com estabilidade mecânica mas com dimensões superiores às necessárias para a cicatrização óssea primária. Através das análises histológicas, nos tempos de implantação escolhidos, foi possível observar todos os passos na cicatrização óssea – fase de reabsorção, fase de modelação e de remodelação (Glimcher, Shapiro et al. 1980, Shapiro 1988, Griffon 2005, Shapiro 2008, Dwek 2010). A fase de reabsorção representa o início da cicatrização onde os osteoclastos são ativados para removerem os detritos e limparem os bordos do defeito após a lesão (Griffon 2005). Nos nossos estudos foi observada a presença de lacunas de absorção, no osso não intervencionado, na zona dos bordos do defeito nas amostras com tempos de implantação mais curtos (30 e 60 dias) (**Figura 11**). Estas lacunas de absorção no osso não intervencionado diminuíram à medida que o tempo de implantação aumentava (**Figura 12**). Curiosamente, nas análises histológicas, observou-se mais lacunas de reabsorção no osso não intervencionado, nos casos em que existia uma maior regeneração óssea (**Figura 13**). Este achado pode ser explicado, por nestes

casos com maior regeneração óssea, existir uma maior atividade celular, tanto dos osteoblastos que sintetizam osso, como dos osteoclastos responsáveis pela reabsorção do tecido ósseo.

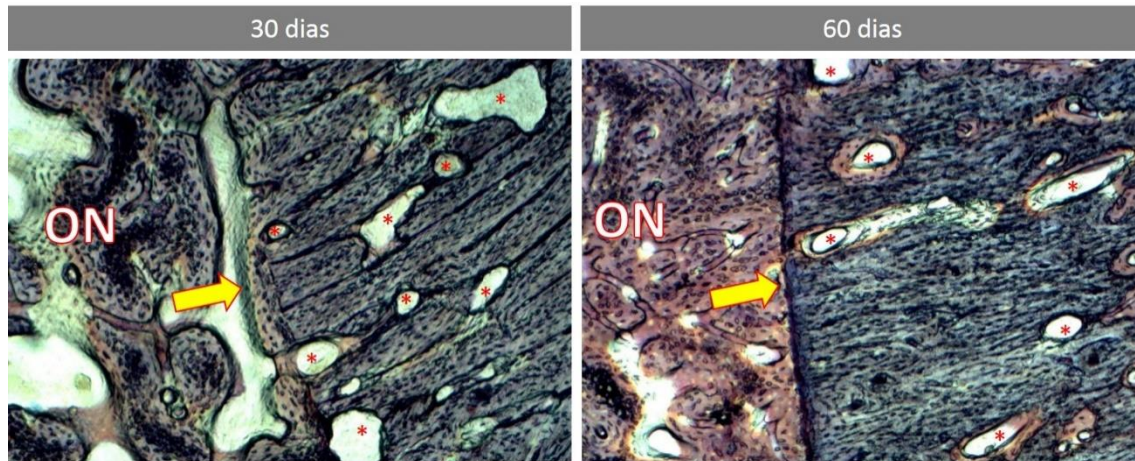


Figura 11 – Fase de reabsorção. Lacunas de reabsorção nos bordos do defeito. ON – osso novo; seta amarela – delimitação dos bordos do defeito; * - lacunas de absorção. Imagens histológicas coradas com Solochrome Cyanine R na ampliação de 400x.

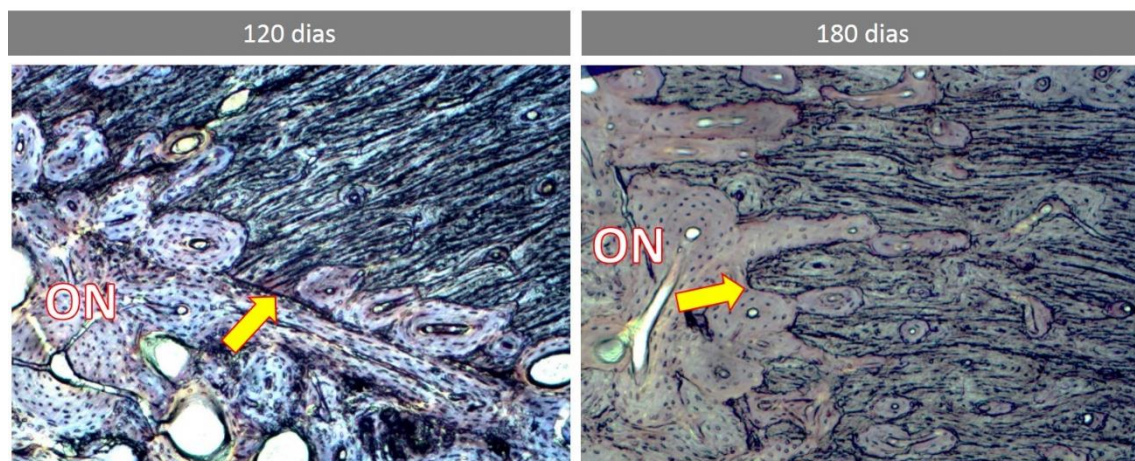


Figura 12 – Diminuição das lacunas de absorção, no osso não intervencionado ao longo do tempo. ON – osso novo; seta amarela – delimitação dos bordos do defeito. Imagens histológicas coradas com Solochrome Cyanine R na ampliação de 400x.

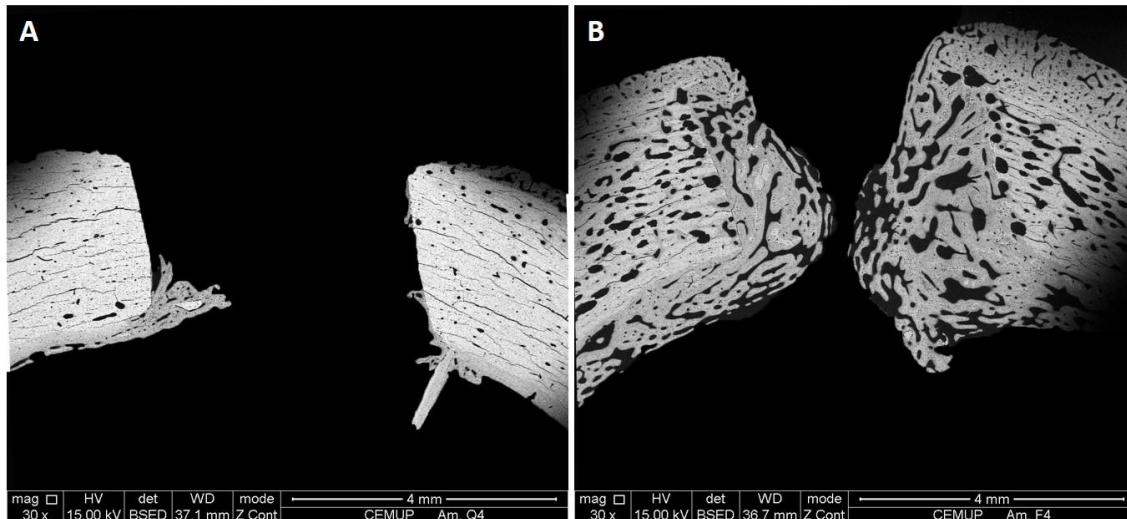


Figura 13 – Lacunas de absorção no osso não intervencionado. Imagens de SEM de controles (defeitos sem biomaterial) com tempo de implantação de 30 dias (A e B). Observam-se mais lacunas de absorção no osso não intervencionado na amostra onde houve um crescimento de osso mais acentuado.

No tempo de implantação de 30 dias, nas amostras sem biomaterial, verificou-se que os osteoblastos sintetizaram fibras de colagénio num raio de 360°, de uma forma desorganizada, para formarem osso primário e que este osso neoformado apresentava um padrão trabecular, principalmente na zona mediana do defeito, com pouca quantidade de matriz óssea e um grande número de osteócitos aglomerados e próximos uns dos outros (**Figura 14**). Este osso, tipo osso primário inicial, serve como suporte estrutural para progressão da cicatrização óssea (Shapiro 2008, Reis, Borges et al. 2010). Com o evoluir do processo de cicatrização, iniciou-se o processo de modelação onde o osso se organiza e adquire uma conformação lamelar. Como o crescimento vascular inicial, neste tipo de defeito, provém da zona medular, a formação deste primeiro osso lamelar segue essa configuração, adotando uma orientação perpendicular ao eixo do osso (**Figura 15**) (Shapiro 2008). Durante este processo de modelação o osso imaturo transforma-se em osso lamelar e começa a compactar (**Figura 15**). À medida que o tempo de implantação aumenta com a progressão da produção de matriz óssea, os osteócitos ficam rodeados por uma maior quantidade de matriz óssea e a distância entre eles aumenta (**Figura 16**). No tempo de implantação de 180 dias, o número e a distância entre os osteócitos atingiram o padrão do osso normal (**Figura 17**). Neste tempo de implantação observou-se o início do processo de remodelação, onde o osso lamelar, formado

originalmente com uma orientação perpendicular ao eixo longitudinal do osso, adotou uma configuração paralela a este eixo (**Figura 18**).

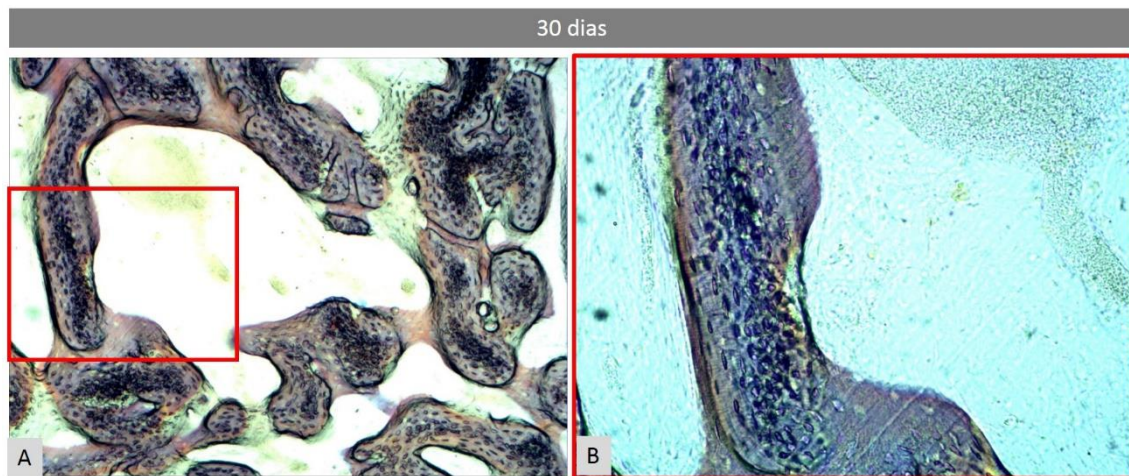


Figura 14 – Osso primário com os osteócitos aglomerados e com um padrão trabecular. Imagens histológicas, de controles com tempo de implantação de 30 dias, coradas com Solochrome Cyanine R na ampliação de 400x (A) e 1000x (B).

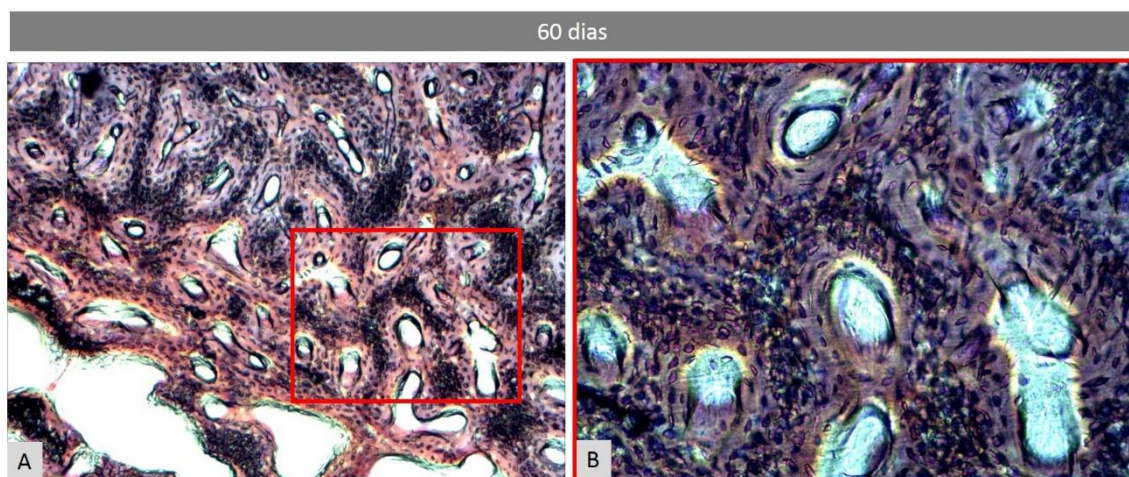


Figura 15 – Processo de modelação. O osso adquire uma conformação lamelar perpendicular ao eixo do osso. Osteócitos aglomerados. Imagens histológicas, de controles com tempo de implantação de 60 dias, coradas com Solochrome Cyanine R na ampliação de 400x (A) e 1000x (B).

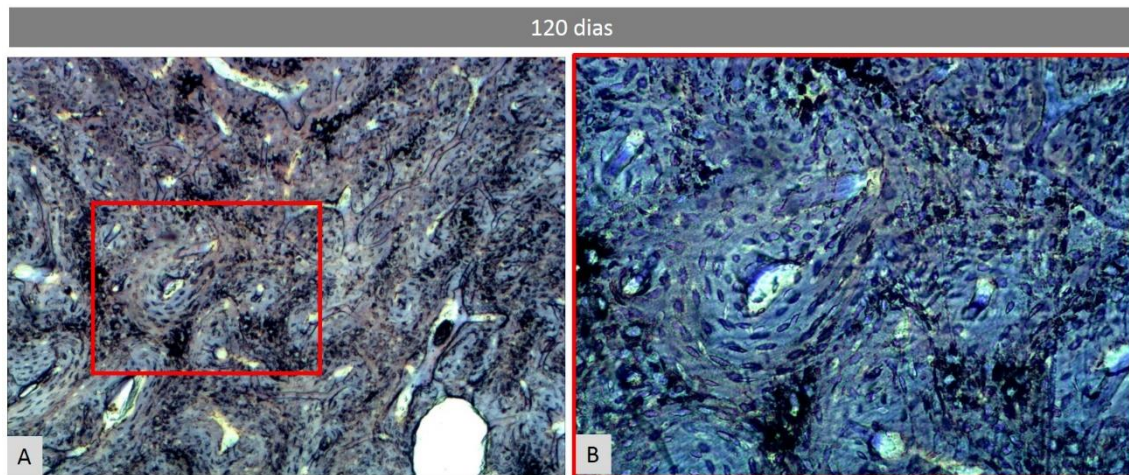


Figura 16 – Com o aumento da produção de matriz óssea, os osteócitos ficam rodeados por uma maior quantidade de matriz óssea e a distância entre eles aumenta. Imagens histológicas, de controles com tempo de implantação de 120 dias, coradas com Solochrome Cyanine R na ampliação de 400x (A) e 1000x (B).

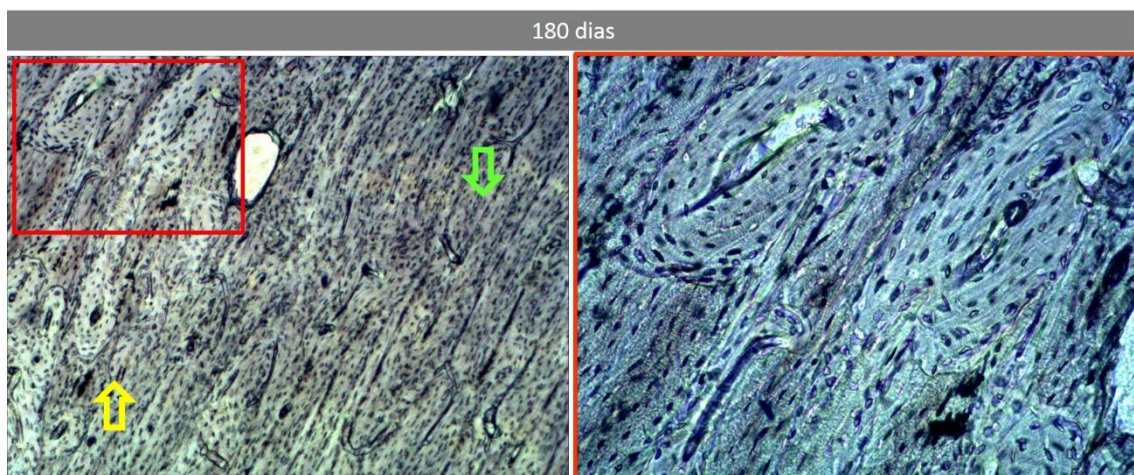


Figura 17 – Remodelação óssea. O padrão lamelar perpendicular ao eixo do osso (seta amarela) adota uma configuração paralela ao eixo do osso (seta verde). Com a progressão da cicatrização óssea, o número e a distância entre os osteócitos atinge o padrão do osso normal. Imagens histológicas, de controles com tempo de implantação de 180 dias, coradas com Solochrome Cyanine R na ampliação de 400x (A) e 1000x (B).

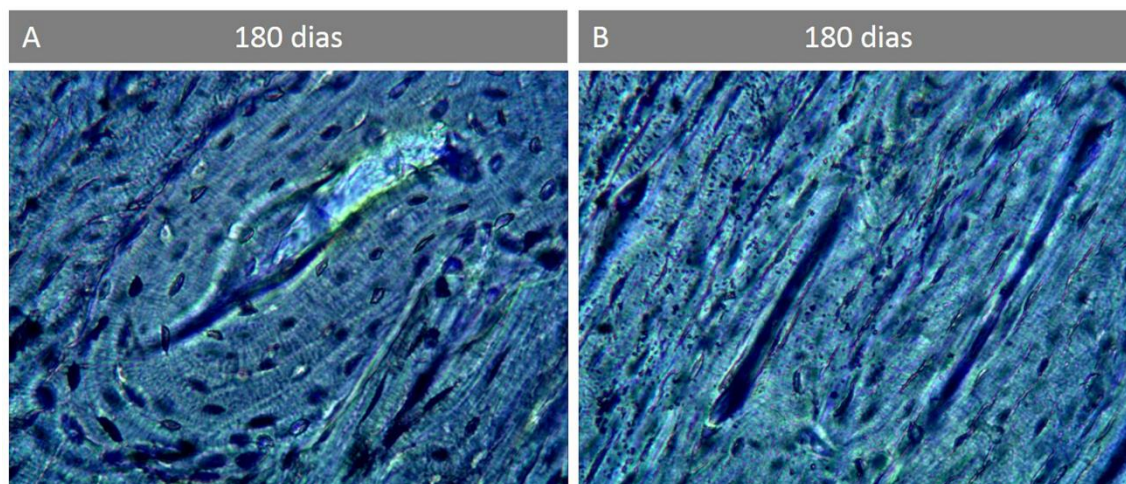


Figura 18 – Osso lamelar com uma orientação perpendicular ao eixo do osso (A). Osso lamelar com uma orientação paralela ao eixo do osso (B). Imagens histológicas, de controles com tempo de implantação de 180 dias, coradas com Solochrome Cyanine R na ampliação de 2000x.

No defeito criado no trabalho desenvolvido aqui descrito, também se verificou a formação de calo ósseo tanto do lado medular, como do lado do periósteo, que se estendeu para além do defeito, para assim aumentar a estabilidade do local da lesão (**Figura 19**) (Griffon 2005, Shapiro 2008, Marsell and Einhorn 2011). O calo medular desenvolveu-se mais rapidamente do que o calo do periósteo (**Figura 19**), pois no processo de cicatrização óssea, o início da formação de osso novo está, numa primeira fase, associada ao desenvolvimento de uma rede vascular proveniente da cavidade medular, com o fornecimento de células da medula óssea ao local da lesão (Shapiro 1988, Shapiro 2008). Durante o processo de remodelação, o calo ósseo é absorvido para o osso adquirir o seu formato original. Esta etapa é a fase mais demorada da cicatrização, podendo demorar bastante tempo a finalizar (Doblaré, García et al. 2004, Marsell and Einhorn 2011). Os nossos resultados também sugerem que o processo de remodelação com a reabsorção do calo ósseo, se inicie no lado medular. A face medular apresenta uma maior atividade e metabolismo quando comparada com a face periosteal, além disso tem uma maior exposição às citocinas provenientes do compartimento medular (Clarke 2008) e a maioria dos osteoclastos têm origem nas células precursoras hematopoiética monocíticas-macrofágicas da medula óssea (Doblaré, García et al. 2004, Clarke 2008). O defeito criado neste modelo apresenta um tamanho não crítico, em que aos 60 dias todos os defeitos apresentavam união óssea, permitindo assim em tempos de implantação curtos avaliar o comportamento dos biomateriais

testados. De referir que tempos de implantação mais alargados permitem a avaliação da taxa de degradação do biomaterial.

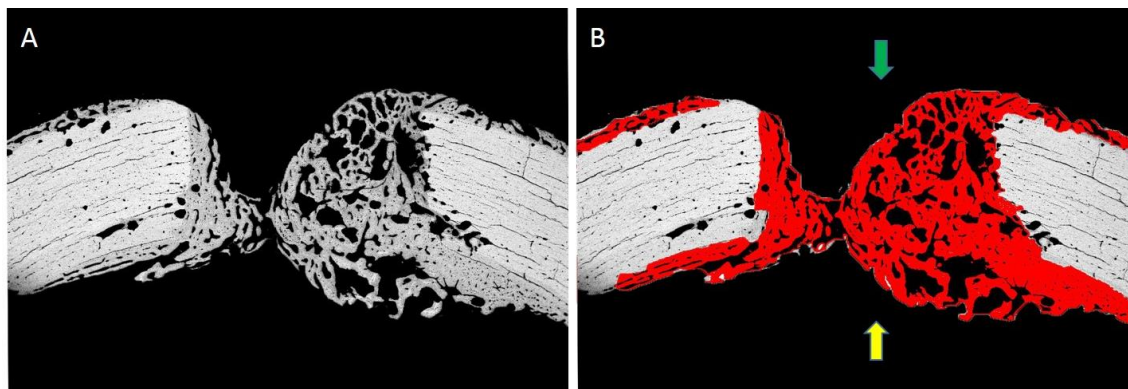


Figura 19 – Calo ósseo. Imagem de SEM com ampliação de 30x de um controlo com tempo de implantação de 30 dias (A e B). Osso novo realçado a vermelho (B), seta verde zona do periósteo, seta amarela zona medular.

Nestes estudos ficou provado que é possível, através do método de *Threshold* selecionar e isolar as frações em estudo (zonas por preencher, lacunas, osso e biomaterial), para calcular áreas, permitindo seguir a evolução da regeneração óssea. Através destes parâmetros pode-se monitorizar e avaliar a *performance* de um biomaterial, fazendo comparações dos resultados obtidos pelos diferentes biomateriais e controlos (**Figura 2**). O grau de reabsorção do biomaterial também poderá ser quantificado por este método, comparando a variação da área ocupada pelo biomaterial ao longo dos tempos de implantação. Este método de *Threshold* mostrou ser um método fiável e objetivo para a realização da análise histomorfométrica. Complementarmente à análise histomorfométrica, também se propõe nesta tese, a realização de estudos histológicos. Estes estudos permitem avaliar a evolução da cicatrização óssea, a qualidade do osso novo formado e a interação do biomaterial com o osso formado. Através da análise histológica pode-se classificar o biomaterial como tóxico, quando se forma uma reação de corpo estranho com o aparecimento de necrose tecidular e processos de inflamação extensos; como bioinerte, quando se forma uma fina camada fibrosa na interface entre o osso e o biomaterial, sem reação inflamatória; como bioativo, quando se forma uma ligação direta entre o biomaterial e o osso formado sem intermédio de tecido fibroso; e como bioreabsorvível, quando o material se dissolve / reabsorve e é gradualmente substituído por osso novo (LeGeros, Lin et al. 2003, Kamitakahara, Ohtsuki et al. 2008, Srivastav 2011). Com este tipo de análise, também se pode observar e

caracterizar o tipo de degradação do biomaterial, visualizando a fragmentação do biomaterial (**Figura 20**), a invasão de osso para o interior do biomaterial (**Figura 21**) e a presença de osteoclastos nas lacunas de reabsorção junto ao biomaterial (**Figura 22**). A avaliação da qualidade do osso formado, também permite avaliar a *performance* do biomaterial, comparando as diferentes composições dos biomateriais em estudo entre elas e com os defeitos controle que não foram preenchidos por biomaterial (**Figura 23**).

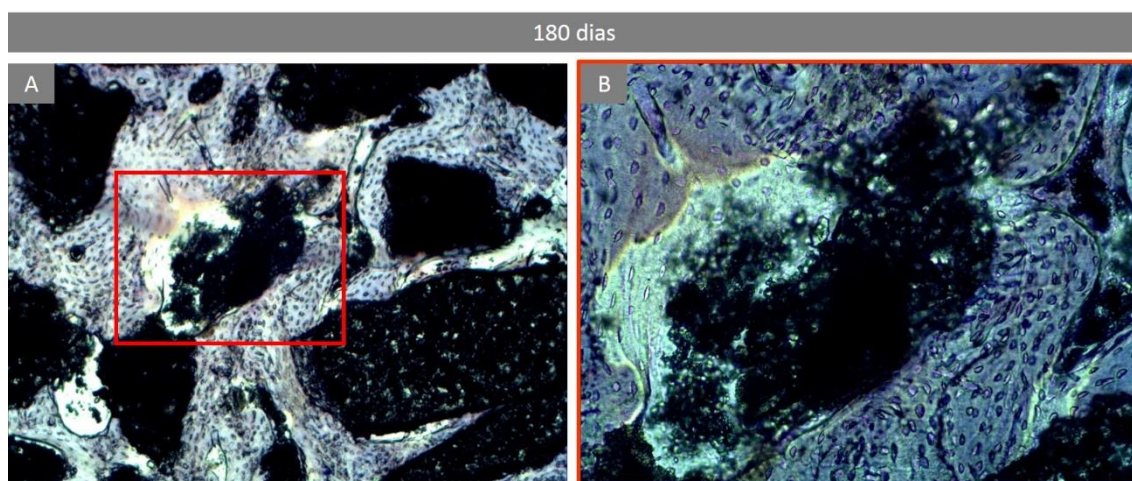


Figura 20 – Fragmentação do biomaterial. Imagem histológica com tempo de implantação de 180 dias de amostra, com Bonelike® poligonal 500-1000µm corada com Solochrome Cyanine R, na ampliação de 400x (A) e 1000x (B).

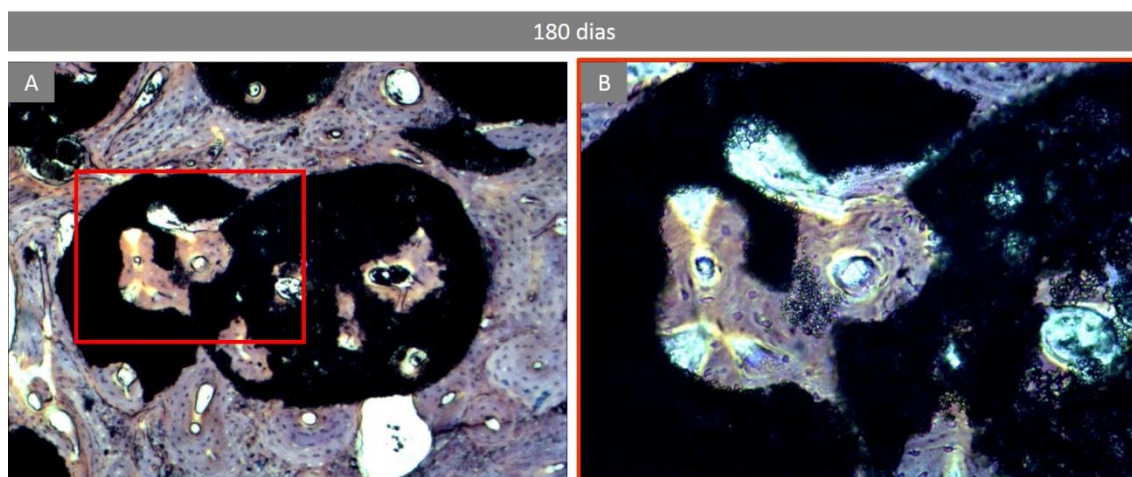


Figura 21 – Invasão de osso para o interior do biomaterial. Imagem histológica com tempo de implantação de 180 dias de uma amostra com Bonelike® esférico 500-1000µm. Imagem corada com Solochrome Cyanine R, na ampliação de 400x (A) e 1000x (B).

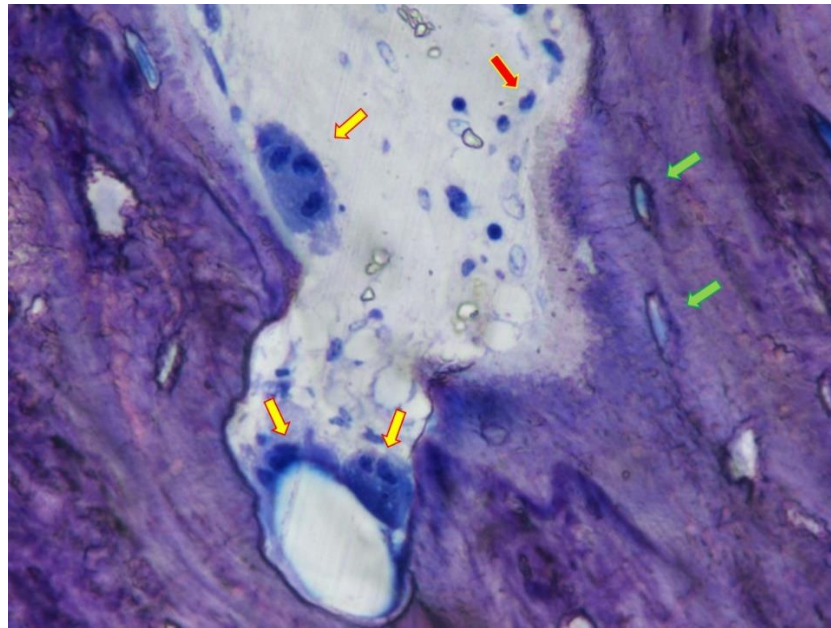


Figura 22 – Osteoclastos (seta amarela), osteoblastos (seta vermelha), osteócitos (seta verde). Imagem corada com Hematoxylin & Eosin, na ampliação de 4000x

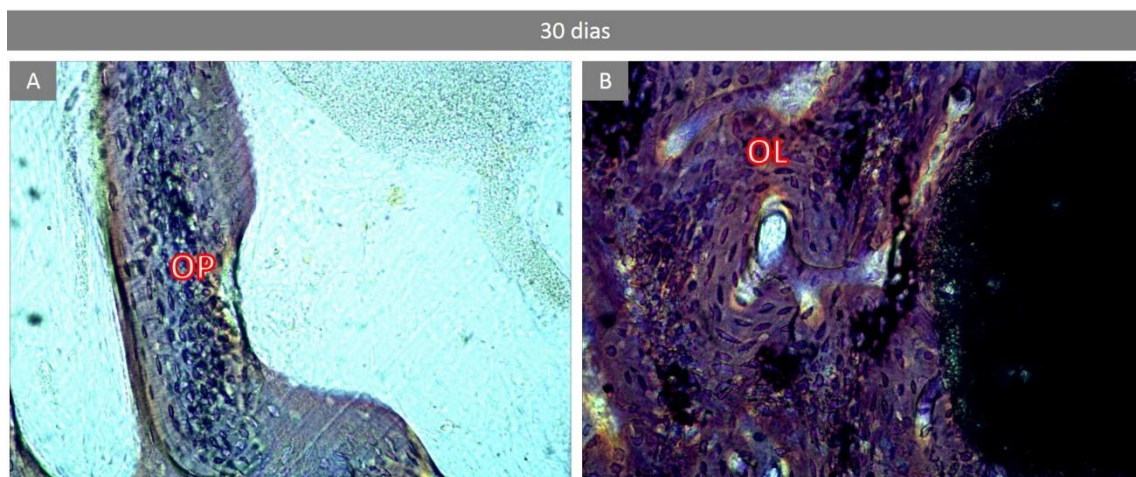


Figura 23 – Imagens histológicas coradas com Solochrome Cyanine R, na ampliação de 1000x, com tempo de implantação de 30 dias de um controle (A) e de uma amostra com Bonelike® 500-1000µm. Osso primário (OP) e osso lamelar (OL).

Nas últimas décadas produziram-se diversos biocerâmicos para funcionarem como substituto ósseo. No desenvolvimento destes biomateriais procura-se otimizar a composição química e encontrar a arquitetura e estrutura ideal para o biomaterial (Hing 2005). A composição química do Bonelike®, com a combinação da HA e as duas fases α e β de FTC, apresenta uma série de vantagens em relação a cada um dos seus componentes isolados. Esta conjugação da HA com os FTC permite criar condições para que o substituto

ósseo seja reabsorvido a um ritmo mais próximo da formação de osso novo. Assim, evita-se o desaparecimento do implante antes da existência de um suporte sólido de osso novo, como sucede no uso isolado dos FTC, e ao mesmo tempo previne-se que a permanência do implante impeça a regeneração óssea, com a consequente fragilização do local da lesão, como acontece no uso da HA isolada (Nery, LeGeros et al. 1992, Brown, McAfee et al. 2010, Yang, Unursaikhan et al. 2013).

A incorporação das duas fases - α e β de FTC - permite um maior controle na taxa de reabsorção do Bonelike®. A reabsorção do implante faz-se assim numa forma gradual, numa primeira fase degrada-se a componente α e posteriormente a componente β , com uma disponibilização progressiva e controlada ao longo do tempo, de iões de cálcio e fósforo, que servem como catalisadores para a formação de osso e precipitação de camadas de apatite adjacente à superfície do material. Esta disponibilização gradual dos iões de cálcio e fosfato permite o fornecimento destes iões em concentrações fisiológicas adequadas, evitando hiperconcentrações que poderiam tornar-se tóxicas e prejudicar a formação de osso novo (Yamada, Heymann et al. 1997, Zaidi, Moonga et al. 2004). Além da reabsorção controlada, a integração do Bonelike® também é fortalecida pelas propriedades bioativas da HA, criando-se ligações diretas entre o osso e o biomaterial, ligações estas catalisadas com a libertação de iões de cálcio e fosfato provenientes da dissolução dos FTC. A incorporação de microelementos como o flúor, o sódio e o magnésio no Bonelike®, permite que estes sejam libertados gradualmente à medida que o implante vai sendo degradado e dissolvido, melhorando assim as condições do microambiente, formado à volta do implante, que favorecem a formação de osso novo (Vasconcelos, Santos et al. 2000, Lopes, Santos et al. 2001, Zyman, Glushko et al. 2004, Kalita, Fleming et al. 2008).

Apesar destas características da composição química do Bonelike®, permitirem a otimização da aplicação como substituto ósseo, ao longo dos tempos de utilização do Bonelike®, surgiram situações que necessitavam de ser melhoradas. Nomeadamente no desenvolvimento da arquitetura do biomaterial para encontrar a estrutura física ideal, tanto para a manipulação como para a osteorregeneração. Inicialmente os grânulos de Bonelike® eram produzidos, numa primeira etapa, em blocos cerâmicos densos, e posteriormente

esmagados isostaticamente, dando origem a um produto final muito heterogêneo de formas poligonais e irregulares com arestas aguçadas. Além do mais, pensa-se que este procedimento de esmagamento destrói a camada superficial, alterando e destruindo a porosidade dos grânulos (Zyman, Glushko et al. 2004).

Assim um dos objetivos deste trabalho foi o desenvolvimento de uma formulação do Bonelike® com uma arquitetura ideal, tanto para a manipulação, como para a estimulação e condução do crescimento ósseo. Numa primeira fase desenvolveu-se e caracterizou-se o Bonelike® com uma forma esférica (*pellets*), no trabalho científico publicado, intitulado “Characterization and preliminary *in vivo* evaluation of a novel modified hydroxyapatite produced by extrusion and spheronization techniques”.

A produção controlada de partículas esféricas (*pellets*) de fosfato de cálcio continua a ser um desafio (Bohner, Tadier et al. 2013). Neste trabalho produziram-se *pellets* de Bonelike®, baseada na tecnologia de extrusão e esferonização farmacêutica (Santos, Lopes et al. 2010). Foi escolhido este método pela sua reprodutibilidade, com uma produtividade de elevado rendimento a um preço bastante acessível. O produto final apresentou um tamanho homogêneo que variava entre os 500 os 1000µm com uma porosidade total de 55,1%, dividida com 24,4% de intraporosidade e 30,7% de interporosidade. A intraporosidade revelou a existência de microporos interconectados com poros de diâmetro menor que 10µm e a interporosidade com poros de diâmetro inferior a 343µm. A presença dos microporos no biomaterial e dos macroporos da interporosidade fornecem condições ideais para o aporte de nutrientes e a remoção de detritos; a ancoragem, proliferação e diferenciação das células; a adsorção de proteínas e o desenvolvimento de uma rede vascular; promovendo assim o crescimento e condução do novo osso, bem como a reabsorção do biomaterial (Hing 2005, Karageorgiou and Kaplan 2005, Bohner 2010, Sanzana, Navarro et al. 2013).

Este estudo preliminar *in vivo*, apesar de ter sido feito somente em 2 tempos de implantação e em 4 animais, apresentou resultados animadores. Mais uma vez provou-se as propriedades osteocondutivas do Bonelike®, com as amostras poligonais e esféricas a apresentarem percentagens de preenchimento do defeito maiores, quando comparadas com os defeitos controle. Este preenchimento ósseo mais efetivo e rápido, diminui o risco do aparecimento de

fraturas pós-cirúrgicas (Benhamou and Roux 2008). Verificou-se também que os defeitos onde foi implantado o Bonelike® esférico (*pellets*) tinham menos espaços vazios, com diferenças estatisticamente significativas, em comparação com os defeitos controle não preenchidos com o biomaterial. Não existindo estas diferenças, com significado estatístico, no caso do Bonelike® poligonal. Estes resultados sugeriram que o Bonelike® esférico poderia apresentar vantagens em relação ao Bonelike® poligonal.

Este estudo preliminar despoletou o interesse para a realização de um trabalho mais alargado, para aprofundamento do conhecimento do comportamento deste novo material. Nomeadamente com a utilização de um número superior de animais e uma diversidade maior de tempos de implantação, para assim se poder analisar mais convenientemente o comportamento *in vivo* do Bonelike® esférico. Neste novo estudo intitulado “Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on osteoregeneration” (Atayde, Cortez et al. 2014) produziu-se um novo tipo de *pellets* com granulometria mais reduzida (250-500µm). Obtiveram-se lotes de Bonelike® poligonal com dimensões 500-1000µm, Bonelike® esférico com dimensões 250-500µm e Bonelike® esférico com dimensões 500-1000µm, com formulação química semelhante, para assim se poder estudar o efeito da forma e granulometria do material na regeneração óssea. O comportamento biológico de um biomaterial pode ser afetado tanto pela sua composição química, como pelas propriedades físicas (Bohner 2010). As composições químicas calculadas por difração de raio X e pela análise pelo método de Rietveld das 3 formulações do Bonelike® foram semelhantes (esféricos 73%HA + 13% β-TCP + 12% α-TCP e poligonal 76%HA + 14% β-TCP + 10% α-TCP), por conseguinte, quaisquer diferenças no comportamento biológico destas 3 formulações de Bonelike®, seriam devidas às suas variações morfológicas.

A arquitetura de um biomaterial, com a presença de macrocopos, microporos, microporos interconectados, tamanho das partículas e o formato do biomaterial são características físicas que podem afetar o comportamento do biomaterial (Galois and Mainard 2004, Bohner, Galea et al. 2012, Sanzana, Navarro et al. 2013). O tamanho da interporosidade tanto do Bonelike® esférico como no Bonelike® poligonal (100 a 150µm) mostrou dimensões adequadas para o crescimento e proliferação das células (Karageorgiou and Kaplan 2005). No

entanto estas dimensões são inferiores aos 300µm estipulados para facilitar a proliferação vascular (Kuboki, Jin et al. 2001, Karageorgiou and Kaplan 2005). Mas é preciso ter em conta que este tipo de material é reabsorvido e à medida que o fenómeno de reabsorção ocorre, a porosidade aumenta, podendo o valor 300µm não ser o limiar para a proliferação vascular neste tipo material (Galois and Mainard 2004). Quanto à intraporosidade obtiveram-se valores distintos para as três formas de Bonelike® testadas. A forma poligonal apresentou valores de intraporosidade muito reduzida, confirmando-se que o processo de fabrico com esmagamento dos blocos, para se obter a fórmula granular, altera a camada superficial, destruindo a microporosidade dos grânulos (Zyman, Glushko et al. 2004). Como era de esperar devido ao tamanho dos grânulos a intraporosidade do Bonelike® esférico 500-1000µm foi superior à do Bonelike® esférico 250-500µm.

Como Já foi referido, nestes trabalhos foram utilizados defeitos de tamanho não crítico, para garantir uma cicatrização óssea rápida e fiel e poderem ser observados as várias fases da cicatrização óssea. Para satisfazer este objetivo, no trabalho onde se estudou a influência da morfologia do Bonelike® (Atayde, Cortez et al. 2014), foram utilizados vários tempos de implantação. Desde períodos curtos como o exemplo dos 30 e 60 dias, para assim se observar os fenómenos iniciais da cicatrização óssea, bem como tempos mais longos de 90, 120 e 180 dias, para poder permitir estudar fases mais avançadas da cicatrização óssea, assim como os fenómenos de absorção e degradação do biomaterial.

Uma das dificuldades encontradas, no modelo animal utilizado, foi a manutenção dos grânulos de biomaterial no local do defeito sem caírem para a medula óssea. Para ultrapassar este problema, recorreu-se á utilização de sangue autólogo sem anticoagulante como veículo. Misturou-se o sangue com o biomaterial e aguardou-se pela formação de um coágulo, até se obter a viscosidade desejada. Durante a aplicação do biomaterial, os defeitos foram impactados com esta mistura, até se assegurar que existia um preenchimento completo do defeito com o biomaterial (**Figura 24**). Inicialmente através do exame radiográfico e posteriormente pelas análises histológicas, verificou-se a correta implantação do biomaterial, apesar de se notar a existência de algum material na zona medular.

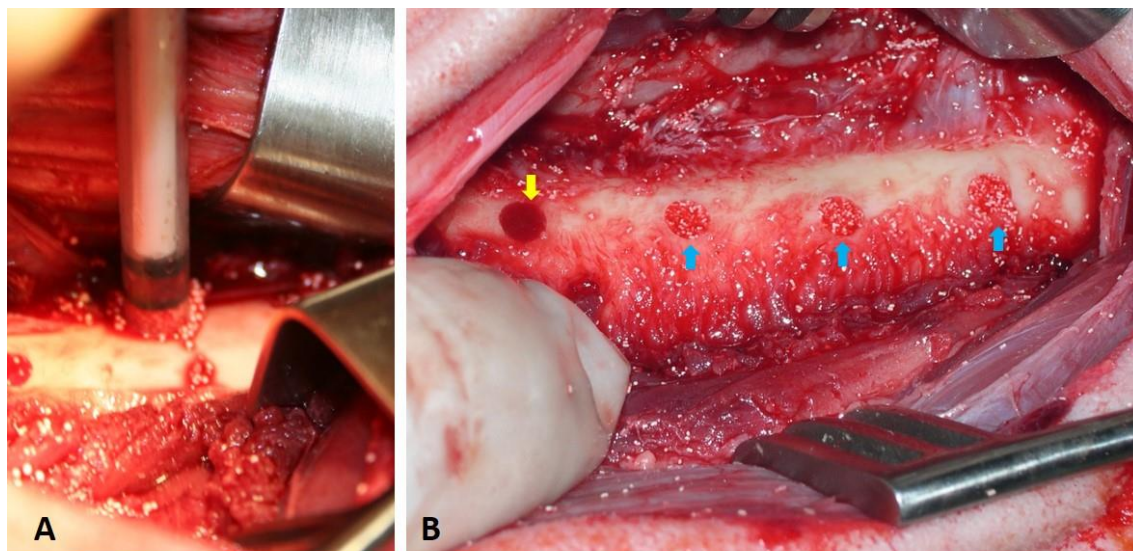


Figura 24 – Implantação do Bonelike® no fêmur de uma ovelha com auxílio de uma seringa (A). Defeito sem preenchimento de biomaterial (defeito controle) (seta amarela); defeitos impactados com Bonelike® (setas azuis) (B).

O uso de blocos, com a geometria idêntica ao defeito (Steffen, Stoll et al. 2001, van Hemert, Willems et al. 2004, Gutierrez, Dias et al. 2007), que se adaptam perfeitamente ao local do implante, poderá ser considerado a situação ideal (Bohner, Tadier et al. 2013), mas infelizmente, na maioria das situações clínicas, principalmente as traumáticas, estes blocos poderão não estar preparados com uma geometria idêntica ao defeito, ao passo que, as formas granulares podem adaptar-se a qualquer tipo de lesão independentemente do formato do defeito (Ribeiro, Barrias et al. 2006).

Na utilização das formulações granulares mantêm-se um espaço entre as partículas (porosidade interpartícula). Este espaço permite a proliferação e crescimento de uma rede vascular, promovendo assim a formação de osso novo e a reabsorção do biomaterial. (Bohner, Tadier et al. 2013).

Nos últimos tempos têm sido desenvolvidas técnicas, para aplicação de enxertos ósseos com procedimentos minimamente invasivos, através de acessos cirúrgicos com incisões pequenas e pouca manipulação de tecidos. Assim sendo, a possibilidade de aplicar o biomaterial através de uma seringa torna-se uma vantagem (Dupraz, Delecrin et al. 1998, Temenoff and Mikos 2000, Bohner 2010, Chen, Zhang et al. 2012, Trombelli, Franceschetti et al. 2012, Gentile, Weinert et al. 2013). Uma das motivações para o desenvolvimento do Bonelike® esférico, foi o melhoramento da injetabilidade deste biomaterial. Vários autores referem que é possível melhorar a injetabilidade, através da utilização

de formas esféricas, pois este tipo de formato facilita a fluidez do biomaterial na extrusão, estando os grânulos menos propensos a compactarem (Ishikawa 2003, Ishikawa, Matsuya et al. 2004, Oliveira, Barrias et al. 2008, Bohner, Tadier et al. 2013). Realmente nos nossos trabalhos, a aplicação de Bonelike® através de uma seringa foi facilitada no caso das formulações esféricas (Bonelike® esférico 250-500 e Bonelike® esférico 500-1000). Já com o Bonelike® poligonal (Bonelike® poligonal 500-1000), verificou-se um efeito de compactação com entupimento no final da seringa, sendo necessária uma força maior para a extrusão do biomaterial.

Como esperado, devido à composição química do Bonelike® com fosfato de cálcio, os defeitos preenchidos com este biomaterial foram observados, no exame radiológico, como círculos radiopacos, enquanto os defeitos vazios, que serviam como controlos, apresentaram-se como círculos radiolucentes. Nos defeitos com Bonelike® nos tempos de implantação mais curtos observou-se uma linha radiolucente à volta do defeito (**Figura 25 A e B**). Este halo radiolucente à volta do defeito poderá representar a fase inicial da regeneração óssea, onde se verifica a atividade dos osteoclastos para removerem detritos dos bordos ósseos, com aumento das lacunas de reabsorção (Griffon 2005). As análises histológicas, dos nossos trabalhos, confirmam a presença de lacunas de absorção nos bordos do defeito, no osso que não foi intervencionado, tanto nos controlos, como já tinha sido observado no estudo que caracteriza o modelo animal usado (Atayde, Cortez et al. 2014), como nas amostras com biomaterial (**Figura 25**). Com o aumento do tempo de implantação tanto o halo radiolucente à volta do implante, das imagens radiográficas, como as lacunas de reabsorção à volta do defeito no osso não intervencionado, das imagens histológicas, vão atenuando e desaparecendo, devido à diminuição dos processos de reabsorção e ao incremento dos processos de regeneração.

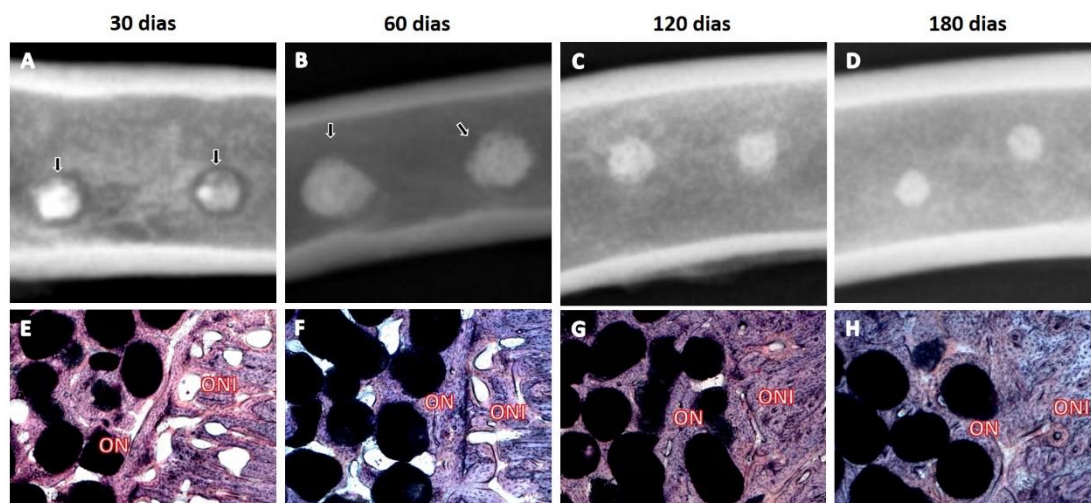


Figura 25 – Reabsorção óssea nos bordos do defeito. Radiografias dos defeitos na projeção lateral (A - D); Imagens coradas com Solochrome Cyanine R, na ampliação de 400x de defeitos preenchidos com Bonelike® 250-500 (E - H). ON – osso novo; ONI – osso não intervencionado. Halo radioluciente à volta dos defeitos observados nos raios X com projeção lateral - seta preta (A e B). Lacunas de absorção no osso não intervencionado (E e F).

Nas análises histológicas pôde-se constatar uma grande variabilidade entre animais. Esta observação foi mais notória nos tempos de implantação mais curtos, devido ao carácter não crítico do defeito. No tempo de implantação de 30 dias certos animais quase que não apresentaram crescimento ósseo, enquanto noutros animais o crescimento ósseo foi suficiente para preencher o defeito. Neste tipo de modelo é muito importante comparar a evolução da cicatrização em defeitos criados no mesmo indivíduo. Nos nossos trabalhos todas as ovelhas tinham defeitos a servir como controlo e ao analisarmos criteriosamente as imagens histológicas, observámos que mesmo em animais onde o crescimento ósseo nos controlos era marginal, os defeitos preenchidos com Bonelike® apresentavam crescimento ósseo suficiente para fazer ponte óssea entre os bordos do defeito, comprovando assim a osteocondução do Bonelike® (**Figura 2 e Figura 3**). Como foi referido, através das análises histológicas do modelo animal utilizado nos nossos trabalhos, foi possível visualizar as várias etapas da cicatrização óssea, permitindo assim comparar entre as amostras o grau de regeneração e maturação óssea, para avaliar a performance das diversas formas de Bonelike® testadas (**Figura 26**). As amostras com Bonelike® esférico evidenciaram uma maturação óssea mais rápida, com a formação de osso lamelar e sistemas de Harvest mais precoce (**Figura 26**). Este fenómeno pode ser um reflexo da maior intraporosidade do material esférico, facilitando assim a

ancoragem das células e a absorção de proteínas, com uma consequente melhor osteocondução (Wozney, Rosen et al. 1988, Wang, Rosen et al. 1990, Osborn 1991, Fleet, Cashman et al. 1996, Riley, Lane et al. 1996, Volek-Smith and Urist 1996, Lin, Su et al. 1997, Okumura, Ohgushi et al. 1997, Yang, Yuan et al. 1997, Carreira, Lojudice et al. 2014). As diferentes formas de Bonelike® mostraram uma boa osteointegração, com a formação de osso novo à volta das partículas de biomaterial, logo no tempo de implantação de 30 dias, e os osteócitos organizados na periferia e à sua volta (**Figura 26 e Figura 27**).

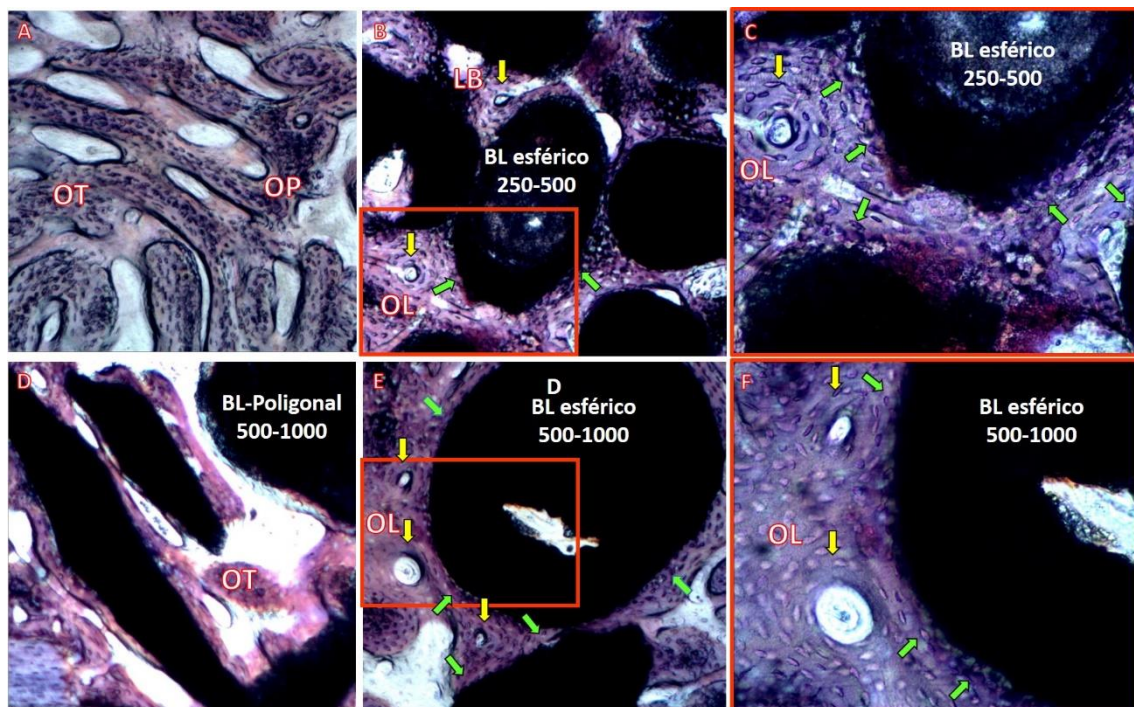


Figura 26 – Comparação da performance do Bonelike®. Imagens coradas com Solochrome Cyanine R, na ampliação de 400x (A, B, D e E) e na ampliação de 1000x (C e F), com tempo de implantação de 30 dias. Defeito controlo sem biomaterial (A); Bonelike® esférico 250-500 (B e C); Bonelike® poligonal 500-1000 (D); Bonelike® esférico 500-1000 (E e F). OT – osso trabecular; OP – osso primário; OL – osso lamelar; seta amarela – sistema de Harvest; seta verde – osteócitos a rodear as partículas de biomaterial.

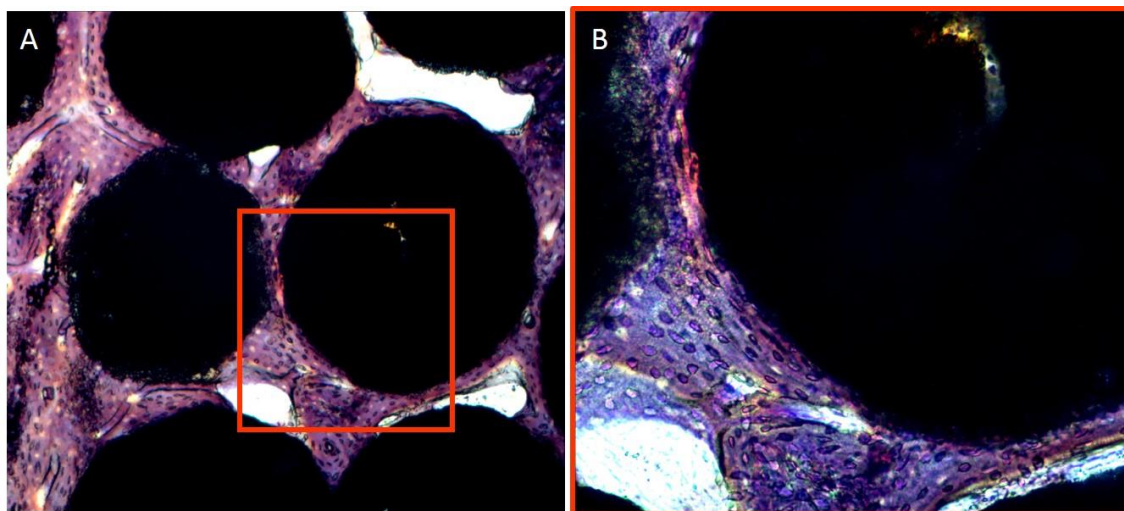


Figura 27 – Osteócitos a rodear as partículas de Bonelike®. Imagem histológica corada com Solochrome Cyanine R, na ampliação de 400x (A) e 1000X (B), com tempo de implantação de 30 dias.

A reabsorção de um biomaterial pode ocorrer por dissolução, reabsorção celular, decomposição enzimática (Yuan, Li et al. 2000, Bohner, Galea et al. 2012), desintegração, fragmentação e invasão óssea (Daculsi, Passuti et al. 1990). No caso do Bonelike® esférico 500-1000µm observou-se um padrão de reabsorção com invasão de osso novo para o interior da partícula. Inicialmente, notou-se um aumento da intraporosidade, com formação de espaços vazios no interior das partículas (**Figura 28**), confirmando-se que em materiais absorvíveis o tamanho da porosidade aumenta ao longo do tempo, devido aos processos de reabsorção (Galois and Mainard 2004). Com o aumento do tempo de implantação estes espaços vazios são substituídos por osso (**Figura 29**). Nos tempos de implantação mais longos observou-se que as partículas, de Bonelike® esférico 500-1000µm, apresentavam bastante osso no seu interior, sendo detetado osso lamelar com os seus sistemas de Harvest (**Figura 21**). Este padrão de invasão do Bonelike® por osso foi menos visível no Bonelike® esférico 250-500µm e raramente observado no Bonelike® poligonal 500-1000µm (**Figura 30**). O Bonelike® esférico 500-1000µm é a formulação com uma intraporosidade maior, facilitando assim a invasão óssea e a degradação do material (Bohner 2010, Sanzana, Navarro et al. 2013). Nas amostras dos defeitos, que continham Bonelike® poligonal, em vez da invasão óssea, observou-se uma fragmentação extensa com formação de múltiplas partículas pequenas (**Figura 31**). Durante a realização do trabalho da caracterização e do estudo preliminar do Bonelike®

esférico 500-1000 μm (Cortez, Atayde et al. 2011), postulou-se que a causa da extensa fragmentação do Bonelike[®] poligonal era devida à sua composição química, com uma percentagem superior de α -FTC e inferior de β -FTC em relação ao Bonelike[®] esférico 500-1000. Como a fase α -FTC é menos estável e mais rapidamente reabsorvida, ponderou-se que este aumento da taxa de degradação resultasse na extensa fragmentação. Este facto não se confirmou com a realização do trabalho onde se estudou o efeito da morfologia do Bonelike[®] (“Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike[®]) on osteoregeneration”) (Atayde, Cortez et al. 2014), pois neste estudo as composições químicas das formulações estudadas eram semelhantes e a extensa fragmentação do Bonelike[®] poligonal manteve-se. O formato poligonal apresenta uma intraporosidade muito baixa, sendo aparentemente a reabsorção feita por dissolução, degradação e fragmentação em vez da invasão óssea observada no caso do formato esférico. A extensa fragmentação com a formação de múltiplas partículas poderá ser prejudicial ao crescimento ósseo, especialmente quando essas partículas são menores que 10 μm (Pioletti, Takei et al. 2000, Böhner, Galea et al. 2012). Outro ponto a salientar, refere-se ao facto da mobilidade das partículas poderem afetar negativamente o desenvolvimento dos vasos sanguíneos e a consequente formação de osso novo (Reis, Borges et al. 2010). No formato poligonal, estão presentes arestas aguçadas que são mais traumatizante para os tecidos envolventes, potencializando o efeito nefasto do movimento das partículas e influenciando negativamente o crescimento ósseo e a reabsorção do biomaterial, estimulando localmente a inflamação. Realmente, no trabalho experimental descrito nesta tese, nas amostras poligonais, verificou-se que as partículas com arestas aguçadas eram menos degradadas (**Figura 31**). Quanto ao formato esférico, além de não ser tão traumatizante para os tecidos envolventes, também se adapta melhor ao padrão circular do crescimento ósseo.

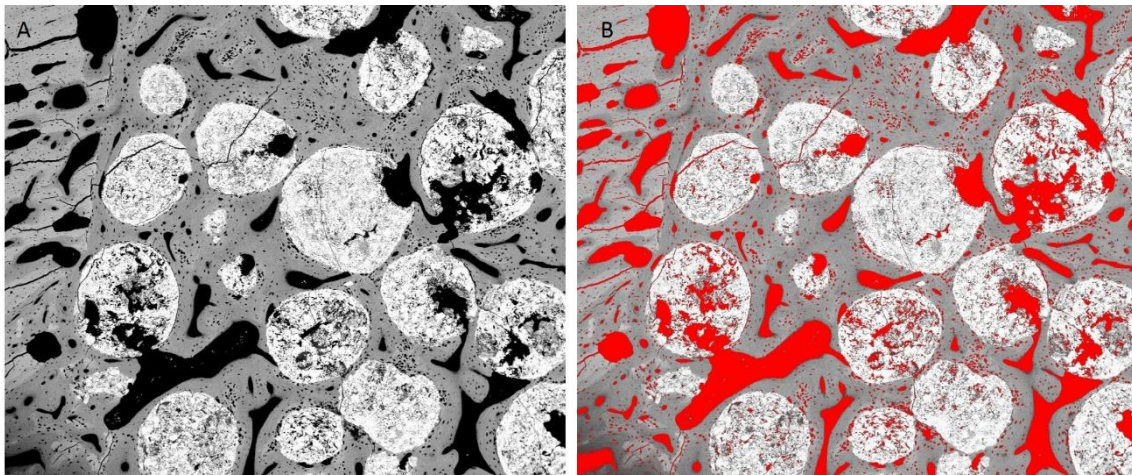


Figura 28 – Aumento da intraporosidade. Imagem de SEM com uma ampliação de 80x com tempo de implantação de 30 dias (A e B). Espaços vazios realçados a vermelho (B).

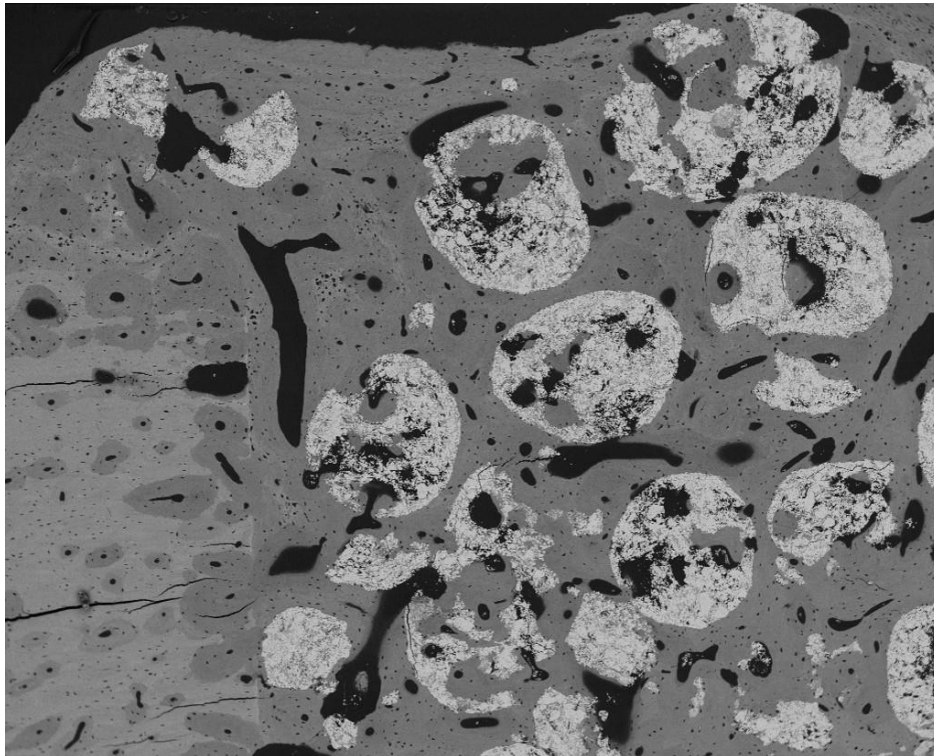


Figura 29 – Formação de osso no interior do Bonelike® 500-1000µm. Imagem de SEM com ampliação de 80x com tempo de implantação de 180 dias.

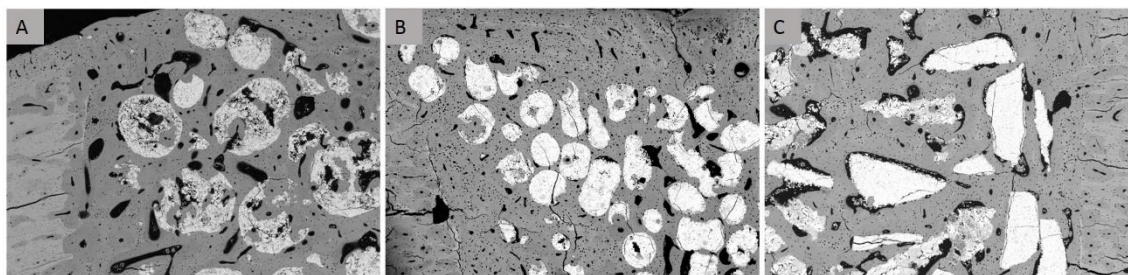


Figura 30 – Invasão de osso para o interior do Bonelike®. Comparação entre as 3 formulações de Bonelike® - Bonelike® esférico 500-1000µm (A); Bonelike® esférico 250-500µm; Bonelike® poligonal 500-1000µm. Imagens de SEM com ampliação de 80x com tempo de implantação de 180 dias.

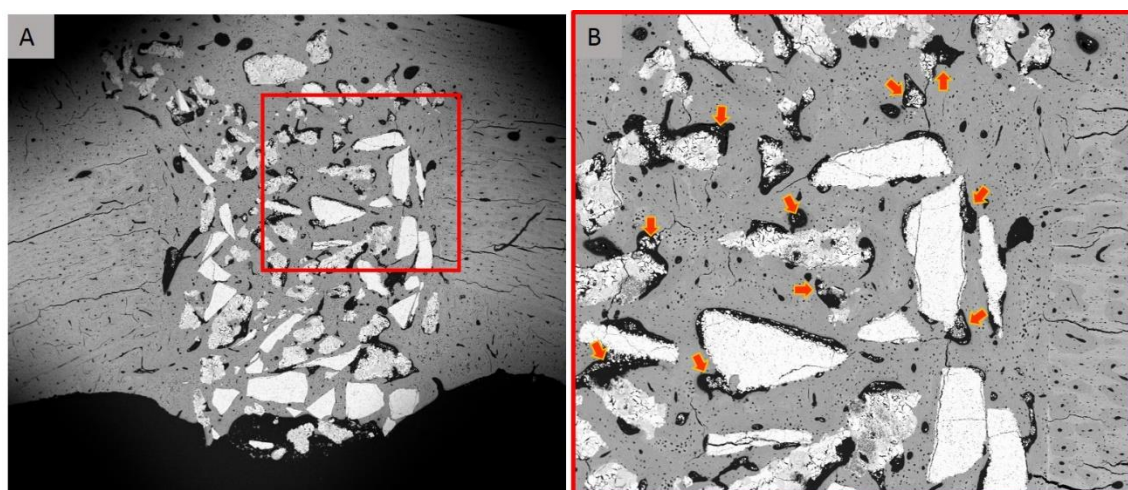


Figura 31 – Fragmentação. Imagem de SEM com ampliação de 30x (A) e 80x (B) de uma amostra implantada com Bonelike® poligonal 500-1000µm no tempo de implantação de 180 dias. Seta vermelha – múltiplos fragmentos pequenos. Nesta imagem observa-se que as partículas com arestas aguçadas são menos degradadas.

A osteocondutividade das diversas formas de Bonelike® estudadas foi confirmada com a presença de pontes ósseas, no tempo de implantação de 30 dias, em todas as amostras com biomaterial, a contrastar com os defeitos de controlo que 61.5% das amostras não realizaram pontes ósseas neste tempo de implantação. Todas as amostras com biomaterial apresentaram pontes ósseas do lado medular, enquanto do lado do perióstio a percentagem de pontes ósseas variou dos 67% (Bonelike® esférico 250-500µm e Bonelike® poligonal) aos 80% (Bonelike® esférico 500-1000µm). Esta incidência maior das pontes ósseas no lado medular é devido à acumulação de maior quantidade de biomaterial do lado medular relativamente à superfície do perióstio. Outra razão para esta observação, poderá ser o crescimento de uma rede vascular proveniente da cavidade medular e da proximidade e exposição às células estaminais e progenitoras hematopoiéticas presentes da medula óssea (Shapiro 1988, Shapiro 2008).

No tempo de implantação mais curto verificou-se que o Bonelike® esférico estimulou o crescimento ósseo e obteve-se um maior preenchimento do defeito com fosfato de cálcio, com uma consequente maior estabilização da zona do defeito. A suportar esta afirmação temos os resultados onde obtivemos uma maior percentagem de osso novo mais Bonelike® (preenchimento com fosfato de cálcio) quando se utilizou as duas granulometrias de Bonelike® esférico, assim como uma percentagem menor de área por preencher do defeito e de espaços vazios. À medida que o tempo de implantação aumenta, a percentagem de osso, nos defeitos controlo foi superior às amostras onde o defeito foi preenchido por Bonelike®, explicando-se este acontecimento, pelo caráter não crítico do defeito criado e pelo efeito do espaço ocupado pelas partículas de biomaterial. Mas ao analisarmos a quantidade de osso novo total em mm² (osso novo do defeito mais osso novo dos calos) (dados não apresentados nos artigos – **Gráfico 1**) a quantidade de osso novo total, nas amostras com biomaterial, foi superior ou semelhante em todos os tempos de implantação, em comparação com os defeitos controlo. No tempo de implantação de 30 dias as formulações esféricas tiveram mais osso total, com diferenças estatisticamente significativas ($p < 0.05$), já no formato poligonal apesar de ter apresentado mais osso as diferenças não foram estatisticamente significativas. A explicação para estas diferenças em relação ao verificado na área que foi definida, para as análises histológicas, como defeito, é a diminuição do efeito da ocupação de espaço pelas partículas de biomaterial, uma vez que a área contabilizada para calcular o crescimento de osso novo total (defeito mais calos) é maior. Comprovamos assim a osteocondução do Bonelike® com a criação de mais osso quando comparada com os defeitos controlo. O efeito da arquitetura do material, na osteocondução também foi provado, pois enquanto as formulações esféricas, quando comparadas com os controlos, apresentaram diferenças estatísticas significativas ($p < 0.05$), com mais osso total, menos área por preencher no defeito e menos espaço vazio no defeito, na formulação poligonal não obtivemos essas diferenças estatísticas.

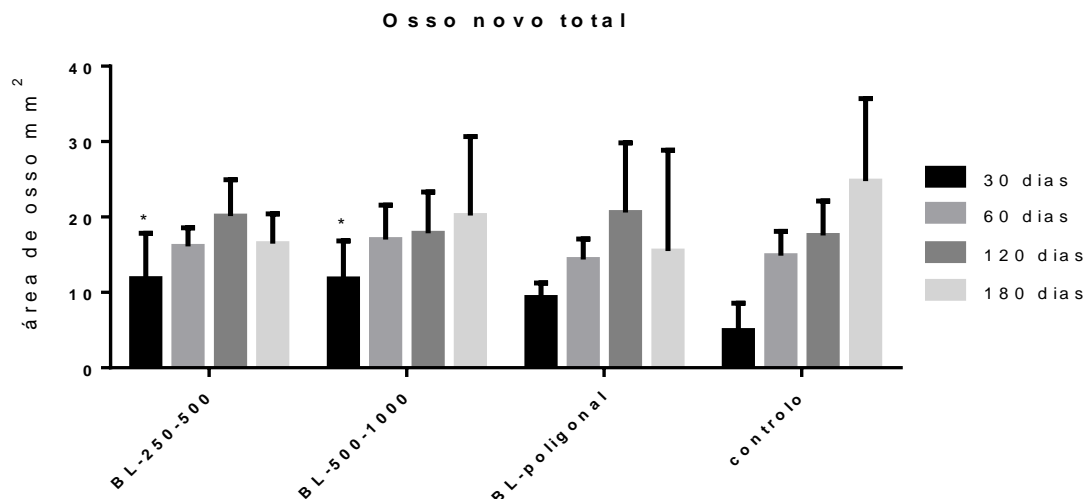


Gráfico 1 – Quantidade de osso novo total (defeito mais calos ósseos), das amostras com os materiais estudados e controles, nos diferentes tempos de implantação. * diferente do controle ($p < 0.05$).

O defeito criado neste modelo, como já foi referido, era um defeito não crítico, com a regeneração óssea a ocorrer mesmo sem intervenção. Por conseguinte, no tempo de implantação de 180 dias em todas as amostras, incluindo os controlos, o defeito estava completamente preenchido. No trabalho onde se estudou o efeito da morfologia do Bonelike® (“Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on osteoregeneration”) (Atayde, Cortez et al. 2014), foram contabilizadas tanto a área por preencher como os espaços vazios (área por preencher mais lacunas) do defeito. A opção da inclusão destes dois parâmetros deveu-se ao facto de no caso da análise da área por preencher isoladamente só nos permite observar a efetiva percentagem de recuperação do defeito, mas não a avaliação direta da fragilidade do osso, isto porque, amostras com áreas por preencher pequenas mas com o osso com um padrão trabecular poderão ser mais frágeis do que amostras com áreas por preencher maiores mas com o osso novo com um padrão mais compacto. Os nossos resultados sugerem que, no processo de cicatrização óssea, o Bonelike® esférico com menor diâmetro (BL esférico 250-500µm), poderá favorecer uma recuperação mais rápida com menores áreas por preencher e menos lacunas. Apesar deste tipo de material ter uma porosidade menor que o Bonelike® esférico 500-1000µm, o menor tamanho do Bonelike® esférico 250-500µm permite uma

área de exposição da superfície maior. Áreas de superfície maiores são benéficas para a osteocondução.

No tempo de implantação de 180 dias, em todos os grupos (controles e biomaterial), as lacunas ósseas atingiram valores abaixo de 11%, que se aproximam da porosidade normal do osso cortical (5 a 10%) (Doblaré, García et al. 2004, Clarke 2008). O valor da percentagem das lacunas ósseas foi semelhante em todos os tempos de implantação, com a exceção do tempo de 180 dias onde BL esférico 500-1000µm e o BL poligonal tiveram mais lacunas do que os controles e o BL esférico 250-500µm. Este acontecimento poderá ser explicado pelo carácter não crítico do defeito e, por outro lado, o osso poderá estar mais reativo, nos grupos com biomaterial, devido aos processos de reabsorção.

Quando se analisa a percentagem de lacunas do osso, como um parâmetro para estudo da cicatrização óssea, terá que se ter em atenção que a presença de menos lacunas poderá não significar um estado mais avançado da recuperação óssea. Por exemplo em amostras em que o defeito ósseo apresenta áreas por preencher elevadas, o osso novo existente vai ser em pequenas quantidades e localizado nas imediações dos bordos do defeito, adjacente às camadas de células mesenquimatosas, que se diferenciam em osteoblastos, com um consequente maior número de osteoblastos com formação de osso mais compacto e com poucas lacunas presentes. Pelo contrário, quando o preenchimento ósseo é elevado, no meio do defeito, afastado dos bordos da lesão e das camadas de células mesenquimatosas, o osso cresce gradualmente adotando inicialmente um padrão trabecular com bastantes lacunas (**Figura 13**). Nos nossos trabalhos, no tempo de implantação de 30 dias, a percentagem de lacunas nas amostras com áreas por preencher maiores foram inferiores às amostras onde as áreas por preencher eram menores.

Durante o processo de regeneração óssea, na fase de reparação, forma-se um calo interior (calo medular) e um calo exterior (calo do córtex), para ajudar a imobilizar o local da lesão. À medida que o processo de cicatrização avança, na fase de remodelação óssea, estes calos são reabsorvidos para o osso voltar a adquirir o formato original (Doblaré, García et al. 2004, Griffon 2005, Shapiro 2008). Devido às propriedades osteocondutivas do Bonelike® verificamos que, nos tempos de implantação mais curtos, o calo medular era maior nas amostras

com biomaterial quando comparadas com os controlos. Além disso o calo medular foi superior ao cortical, devido à acumulação de mais material do lado medial e ao contacto com o a cavidade medular. Verificamos também que o calo nas amostras de Bonelike® poligonal, nos tempos de implantação de 120 e 180 dias, foi superior ao dos outros grupos. Como referido, com a evolução da cicatrização, o calo formado sofre processo de remodelação e é absorvido para o osso adotar o formato original (Doblaré, García et al. 2004, Griffon 2005, Shapiro 2008), a baixa porosidade do material poligonal pode diminuir e retardar este processo de reabsorção.

No desenvolvimento do nosso trabalho também pretendemos aplicar o Bonelike® em situações clínicas de Medicina Veterinária. Este biomaterial foi aplicado em 3 casos clínicos, que tiveram que ser submetidos a artrodeses cirúrgicas (Atayde, Mendes et al. 2011, Atayde, Mendes et al. 2013, Atayde, Mendes et al. 2014). Está descrito, o uso de autoenxertos nas artrodeses cirúrgicas para acelerar o processo, melhorar o contacto e preencher pequenas lacunas entre os dois extremos ósseos (Johnson 1995, Lesser 2002, Sorrel Langley-Hobbs M.A. 2010). Já foram referidas, ao longo deste trabalho, as desvantagens dos autoenxertos, tendo estas desvantagens estimulado o interesse na substituição destes autoenxertos por enxertos ósseos sintéticos (Lopes, Santos et al. 1998, Carson and Bostrom 2007). A utilização do Bonelike®, neste tipo de intervenção, poderá apresentar vantagens em relação aos autoenxertos. O Bonelike®, com as suas propriedades osteocondutivas, pode ser usado na técnica de artrodese para preencher espaços vazios entre a articulação, ajudando na estabilização, promovendo e acelerando o contacto e a fusão óssea. A utilização do Bonelike® foi realizada num caso clínico de um cavalo com uma subluxação da articulação interfalângica proximal, num burro com uma deformidade flexora severa da articulação metacarpofalângica e num cavalo com artrose e instabilidade das articulações intertársica distal e tarsometatársica.

A técnica de artrodese cirúrgica é utilizada em situações clínicas em que não é possível recuperar a articulação, como por exemplo, na doença degenerativa articular, casos de luxações articulares, lesões de ligamentos, algumas fraturas articulares e quando com o tratamento médico convencional não se obtém resultados satisfatórios. Nas articulações das extremidades dos

membros esta técnica cirúrgica tem melhor prognóstico quando é realizada nos membros posteriores (Caron, Fretz et al. 1990, MacLellan, Crawford et al. 2001, Knox and Watkins 2006). Este procedimento permite estabilizar a zona articular, aliviando a dor e a disfunção articular. Os casos das artrodeses da articulação interfalângica proximal e da articulação intertársica distal em conjunto com a articulação tarsometatársica, devido a serem articulações com pouco movimento, permitem que o cavalo recupere a locomoção e atividade normal. Apesar da articulação metacarpofalângica ser uma articulação com movimentos importantes, esta técnica continua a estar indicado para diminuir a dor e aumentar o conforto e uso do membro afetado, prevenindo assim, o aparecimento de lesões de sobrecarga no membro contralateral (Caron, Fretz et al. 1990, MacLellan, Crawford et al. 2001, Knox and Watkins 2006). A técnica de artrodese consiste, na remoção da cartilagem articular para promover o contacto ósseo e a consequente fusão da articulação. Após a remoção da cartilagem articular a articulação deve ser alinhada e estabilizada na posição fisiológica (Caron, Fretz et al. 1990, MacLellan, Crawford et al. 2001, Atayde and Mendes 2004, Zubrod and Schneider 2005, Knox and Watkins 2006, Sorrel Langley-Hobbs M.A. 2010).

O caso clínico onde se realizou a artrodese da articulação interfalângica proximal reporta um cavalo, que após um acidente, fez uma laceração na parte lateral da quartela. Na altura do acidente a laceração foi tratada e suturada. Após a cicatrização da ferida, quando o cavalo retomou a atividade física, o animal durante a locomoção, a passo, evidenciou uma instabilidade da zona da quartela com claudicação. Neste exame dinâmico, além da instabilidade articular, o cavalo elevava a pinça quando apoiava o membro no solo. No exame por palpação dessa região notou-se uma subluxação da articulação interfalângica proximal. O trauma da zona da quartela foi responsável por lesões ligamentares que criaram instabilidade desta articulação. A decisão para a realização da artrodese deveu-se à instabilidade biomecânica com sinais de subluxação, evidenciada no exame clínico, desta articulação. No exame radiológico, na projeção dorso palmar, realizada antes da cirurgia, verificou-se um aumento ligeiro no espaço articular no lado medial. A técnica de artrodese está indicada em situações clínicas de instabilidade articular e, além disso, como a articulação interfalângica proximal apresenta poucos movimentos, o prognóstico para a

recuperação do andamento normal do cavalo é favorável. Acedeu-se à articulação e removeu-se a cartilagem articular com auxílio de uma cureta. Durante a cirurgia confirmou-se que existia um aumento do espaço articular na zona medial da articulação (**Figura 32**). O espaço articular foi preenchido com Bonelike® esférico 250-500µm. Para estabilizar a articulação foram colocados 3 parafusos corticais de compressão. Para se obter a compressão na zona articular, a broca utilizada para a realização do orifício na primeira falange tinha um diâmetro maior (4.5mm) do que o orifício realizado na segunda falange (3.2mm). Posteriormente passou-se um trado de 3.2mm no orifício da segunda falange. Colocaram-se os parafusos, a unir as duas falanges, com o diâmetro igual ao orifício realizado na segunda falange (3.2mm). Este procedimento faz com que os parafusos só façam força na segunda falange, comprimindo assim a segunda falange contra a primeira falange. O recesso realizado na primeira falange tem como objetivo fazer com que as cabeças dos parafusos fiquem adaptadas ao osso sem nenhuma saliência (**Figura 33**). Após a cirurgia, o cavalo ficou com o membro imobilizado, com um aparelho de fibra de vidro durante 90 dias. Quando se removeu o aparelho de imobilização, verificou-se que a instabilidade e a subluxação da articulação interfalângica proximal tinham sido corrigidas. Apesar disto o cavalo ao andar elevava as pinças, provavelmente porque o tendão flexor digital profundo também teria sido afetado na altura da lesão. Esta situação foi corrigida gradualmente com a utilização de ferraduras com extensão nos talões. Durante o seguimento radiográfico verificou-se a formação de osso novo à volta da articulação, com evidências de fusão articular.

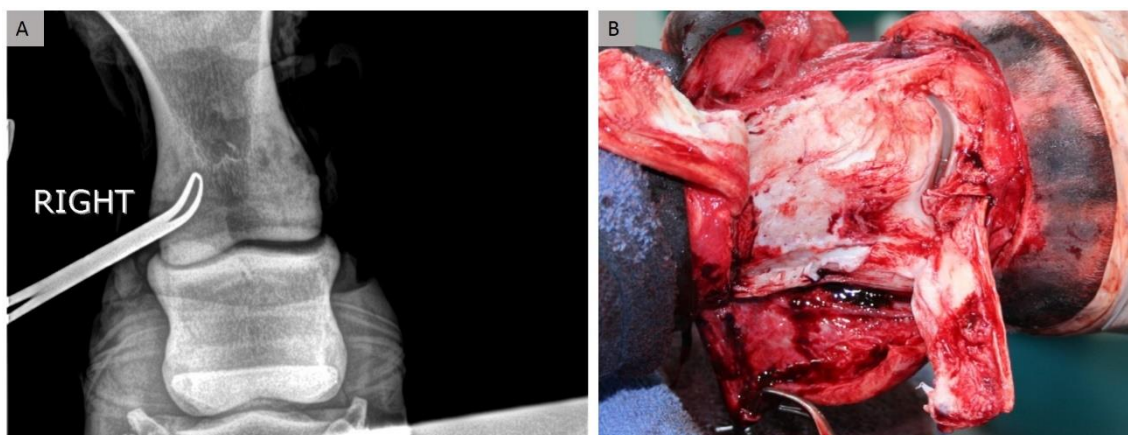


Figura 32 – Zona medial da articulação interfalângica proximal com espaço articular aumentado. Raio X na projeção dorsoplantar (A); fotografia do espaço articular após o acesso cirúrgico (B).

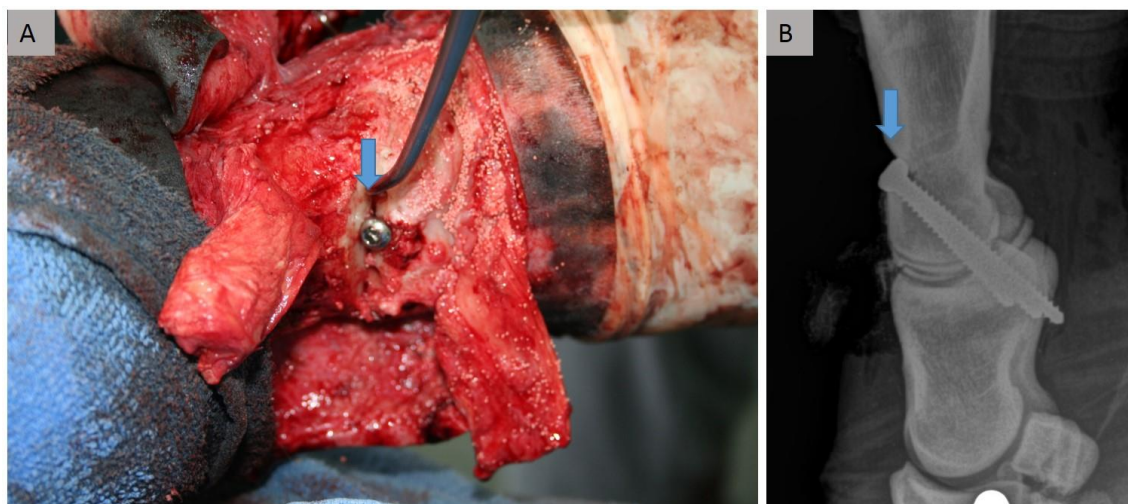


Figura 33 – Recesso realizada na primeira falange para as cabeças dos parafusos não ficarem salientes. Colocação do parafuso durante a cirurgia (A). Raio X pós-operatório na projeção lateral (B). Seta azul – recesso.

O caso clínico onde se realizou a artrodese da articulação metacarpofalângica reporta-se a um burro neonata com uma deformidade flexora congénita severa. A deformidade flexora é uma patologia dos animais em crescimento, onde ocorre um desvio no plano sagital, com uma flexão permanente da zona articular. Esta patologia é normalmente designada por contração de tendões, no entanto este é um termo incorreto, pois na maioria dos casos não se verifica a contração verdadeira dos tendões, mas sim um não alongamento destes ao mesmo ritmo do crescimento ósseo (Kidd and Barr 2002). Estas deformidades podem estar presentes logo na altura do nascimento (congénitas), ou desenvolverem-se ao longo do crescimento (adquiridas) (Auer 2006).

Várias causas são postuladas para o aparecimento das deformidades flexoras congénitas, como o mau posicionamento intrauterino; a presença fetos demasiado grandes em relação à mãe, que dificulta a movimentação dos membros intrauterinamente; fatores teratogénicos; envolvimento de uma componente genética; e a infeção pelo vírus da influenza (Kidd and Barr 2002, Auer 2006). Normalmente as deformidades flexoras são corrigidas com tratamentos conservativos e médicos, sendo indicado o tratamento cirúrgico somente nos casos severos ou que não respondam ao tratamento médico. A palpação das estruturas envolvidas e a manipulação do membro, para determinar o grau de mobilidade e tensão dos tendões, são sinais importantes para a escolha da abordagem terapêutica. Articulações onde se consegue

manipular e estender facilmente o membro não são candidatas ao tratamento cirúrgico. Pelo contrário, nos casos onde não se consegue repor o membro na posição normal e se verifica uma grande tensão nos tendões, deve ser ponderada a opção cirúrgica (Auer 2006). O objetivo da cirurgia é aliviar a restrição à extensão do membro, criada pelos tendões ou ligamentos tensos. Para isso em casos mais ligeiros, recorre-se à desmotomia do ligamento acessório proximal ou distal, consoante esteja o tendão flexor digital superficial ou o tendão flexor digital profundo envolvidos, respetivamente, tendo estas técnicas um bom prognóstico para o futuro uso do cavalo. Em casos mais graves, pode ser necessário recorrer à tenotomia do tendão envolvido, sendo que, este tipo de cirurgia acarreta um prognóstico reservado para o futuro uso do animal (Adams and Santschi 2000, Kidd and Barr 2002, Auer 2006). No caso clínico descrito, o burro apresentava uma deformidade flexora severa. No exame por palpação era impossível manipular e mover a articulação metacarpo falângica. A agravar a situação o animal apoiava a parte dorsal do boleto no solo, tendo já uma úlcera que comunicava com a articulação. Devido à gravidade dos sinais clínicos, e à possibilidade de desenvolvimento de complicações que poderiam agravar a situação clínica, considerou-se o tratamento cirúrgico como primeira opção (Kidd and Barr 2002). A abordagem normal, no tratamento cirúrgico é aliviar a pressão das estruturas que se encontram sobre tensão e contribuem para a deformidade flexora (Adams and Santschi 2000, Kidd and Barr 2002, Auer 2006). Neste caso específico ambos os tendões flexores e mesmo o tendão extensor digital comum, que se encontrava deslocado caudalmente, para a parte lateral do metacarpo, estavam sob tensão e contribuíam para a deformidade flexora. Para se conseguir repor a articulação para a posição fisiológica, estes tendões tiveram que ser cortados (**Figura 34**). Após o corte destas estruturas o membro deixou de ter um suporte estrutural, por isso resolveu-se realizar a artrodese da articulação metacarpofalângica, para estabilizar o membro na posição fisiológica. Apesar de não ser comum a realização da artrodese nos casos clínicos de deformidades flexoras, esta abordagem já foi descrita por Whitehair e colaboradores, que realizou a artrodese desta articulação numa lama, num cavalo miniatura e num burro miniatura com deformidades flexoras severas (Whitehair, Adams et al. 1992). A artrodese da articulação metacarpofalângica não permite a recuperação da atividade normal do animal,

mas neste caso clínico era a única solução que existia, para melhorar a qualidade de vida do Burro. Devido à severidade da situação clínica, com ulceração e exposição articular da parte dorsal da articulação do boleto, o animal apresentou diversas complicações e não recuperou, tendo que ser eutanasiado.

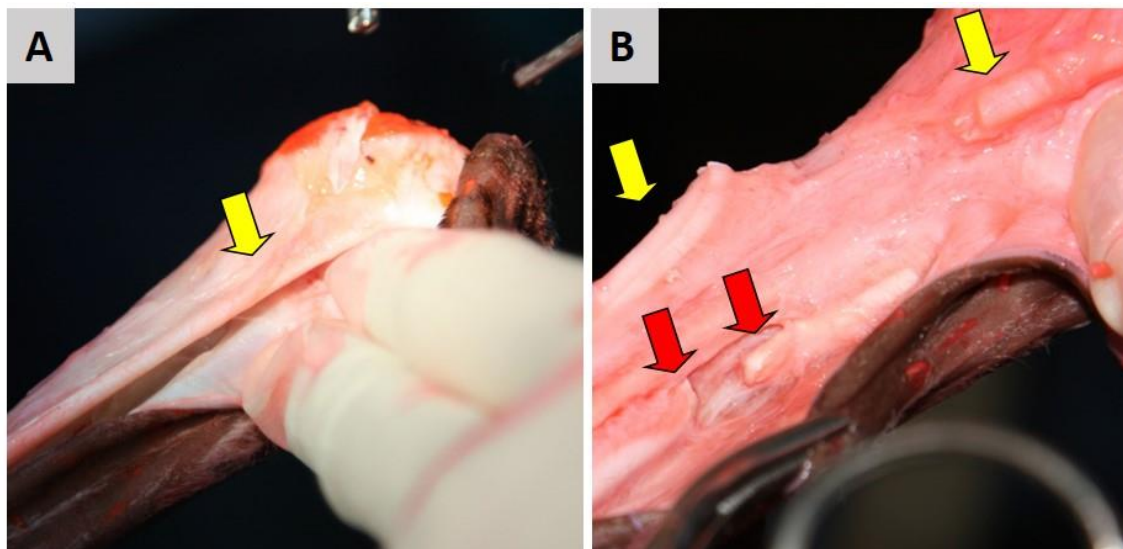


Figura 34 – Tendão extensor digital comum deslocado caudalmente – seta amarela (A). Tenotomia do tendão extensor digital comum (seta amarela), tenotomia dos flexores digitais – seta vermelha (B).

O último caso clínico, onde se realizou a artrodese da articulação intertársica distal em conjunto com a articulação tarsometatársica, reporta um cavalo com esparvão ósseo e consequente claudicação crônica e intermitente do membro posterior direito, com instabilidade biomecânica na região do curvilhão. O esparvão ósseo é uma patologia que apresenta lesões degenerativas na articulação intertársica distal e tarsometatársica (Zubrod, Schneider et al. 2005). As causas mais frequentes para o aparecimento desta patologia são situações que aumentam o *stress* traumático nesta região, como por exemplo a má conformação do curvilhão, o trauma de torsão na região do curvilhão, provocado em certas modalidades equestres (*dressage*, saltos de obstáculos, toureiro, por exemplo) e a concussão excessiva (Zubrod, Schneider et al. 2005). Em certos casos, esta patologia poderá ser autolimitante, com a formação de uma anquilose espontânea e o desaparecimento dos sinais clínicos. A formação de uma anquilose espontânea é incerta, podendo levar muito tempo para acontecer e em alguns casos nunca se chega a formar (Gough, Thibaud et al. 2010). Está descrito a realização da artrodese química destas articulações,

mas este procedimento poderá levar ao aparecimento de diversas complicações, como dor após o procedimento, dano nos tecidos moles e a progressão das lesões degenerativas para as articulações adjacentes (Lesser 2002, Dechant, Baxter et al. 2003, Zubrod and Schneider 2005, Schoenhaus, Lam et al. 2009, Sorrel Langley-Hobbs M.A. 2010). Devido a estas complicações são poucos os clínicos que recorrem à artrodese química (Dechant, Baxter et al. 2003), sendo a artrodese cirúrgica uma melhor alternativa para acelerar a anquilose destas articulações.

Para realizar a artrodese destas articulações passa-se uma broca no plano da articulação, realizando três trajetos através de um ponto de entrada único. O objetivo de passar a broca no plano articular é a destruição da cartilagem articular, promovendo o contacto das extremidades ósseas e a consequente fusão articular (Auer 2006). A colocação do Bonelike® nos orifícios criados permite melhorar a estabilidade mecânica e promover e acelerar as pontes ósseas para consolidar a anquilose da articulação. Teoricamente, quando se realiza uma artrodese deve-se remover a maior quantidade possível de cartilagem, no entanto, neste técnica específica a remoção excessiva de cartilagem causa muita instabilidade e dor pós operatória. Apesar da técnica utilizada não induzir uma artrodese completa, deste procedimento resultam áreas multifocais de anquilose, que estabilizam as articulações distais do tarso e eliminam a fonte de dor e claudicação (Dechant, Baxter et al. 2003). As propriedades osteocondutivas do Bonelike® poderão melhorar e reforçar estas áreas focais de anquilose. Com esta intervenção a situação clínica do cavalo melhorou, permitindo que o cavalo voltasse à atividade competitiva. Em todos os casos clínicos o uso do Bonelike® não criou nenhuma reação adversa local ou sistémica (**Figura 35**).

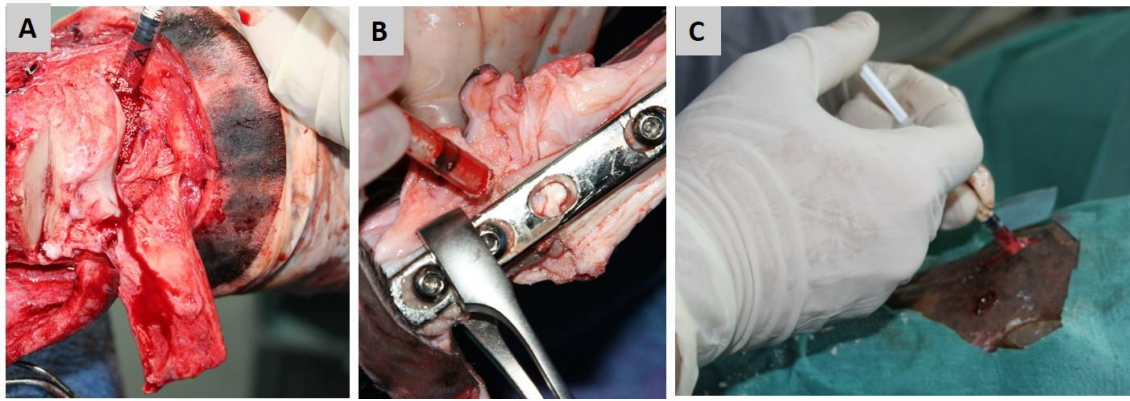


Figura 35 – Colocação de Bonelike® no espaço articular. Articulação interfalângica proximal (A); articulação metacarpofalângica (B); articulação tarsometatársica (C).

Capítulo 7

Conclusões

7 - Conclusões:

Com os resultados apresentados nesta dissertação, podemos concluir, que a utilização do modelo animal proposto permite realizar amostras em série, no mesmo animal e com os mesmos fatores condicionantes, diminuindo assim a variabilidade individual, sendo possível o estudo comparativo de diferentes composições de substitutos ósseos implantados para promoverem a regeneração óssea. Apesar de este modelo apresentar um defeito de tamanho não crítico, foi provado que se consegue avaliar de forma sistemática o comportamento dos biomateriais, sendo ainda possível detetar diferenças de comportamento *in vivo* entre os biomateriais em estudo. Este carácter não crítico dos defeitos também possibilita a observação e a avaliação de todas as fases da cicatrização/regeneração óssea em períodos de implantação curtos. Uma das vantagens do tamanho do defeito, no modelo desenvolvido, como já foi referido, é a possibilidade da análise em série num mesmo animal, controlando-se assim a variabilidade individual e, além disso, permite diminuir ao máximo o número de animais utilizado na experimentação, sem perder a validade estatística. Seguindo este princípio, as diretrizes da política dos 3Rs são aplicadas (*Reduction, Refinement, and Replacement*) (Hansen 2002). Neste trabalho desenvolveu-se ainda um método rápido e reprodutível para a análise histomorfométrica do tecido ósseo regenerado, que consiste na segmentação semiautomática das imagens histológicas. Este novo método de *Threshold* foi validado, comparando-o com o teste usado tradicionalmente nos estudos histomorfométricos descritos nas publicações científicas disponíveis. Com o método desenvolvido é possível seleccionar e medir áreas de osso novo, lacunas biomaterial e zonas por preencher, para assim estudar o comportamento dos substitutos ósseos na regeneração óssea. É sem dúvida, um método rápido, objetivo e reprodutível.

No decorrer dos nossos estudos mais uma vez ficaram provadas as propriedades osteocondutivas do substituto ósseo Bonelike®. Desenvolveu-se uma nova formulação esférica, que mostrou vantagens em relação ao Bonelike® originalmente desenvolvido com o formato poligonal, aumentando a sua injectibilidade. Comprovou-se ainda a influência da forma, tamanho, estrutura e microestrutura no comportamento *in vivo* do Bonelike®. O Bonelike® esférico

evidenciou ter uma microestrutura com porosidades maiores, que favorecem a osteocondutividade, osteointegração e a absorção do biomaterial durante todo o processo de cicatrização / regeneração óssea. O formato esférico tem ainda a vantagem de se adaptar melhor ao crescimento ósseo. O Bonelike® esférico apresenta também uma melhor injectabilidade comparativamente aos formatos poligonais testados, uma vez que apresenta uma maior facilidade de aplicação através de seringa.

O Bonelike® esférico também foi utilizado em casos clínicos de Medicina Veterinária, não mostrando nenhuma reação adversa local ou sistêmica, tendo promovido corretamente e num período de tempo curto, a artrodese das articulações onde foi aplicado. O Bonelike® esférico é um substituto ósseo que pode ser usado em casos clínicos de artrodese para preencher espaços vazios, aumentar a estabilidade articular e para promover a osteorregeneração num período de tempo encurtado melhorando o prognóstico clínico.

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